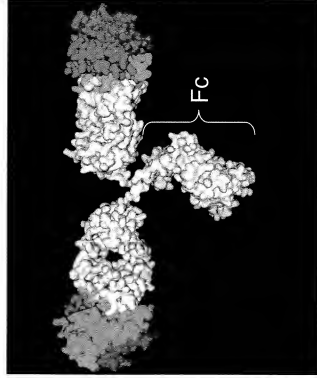


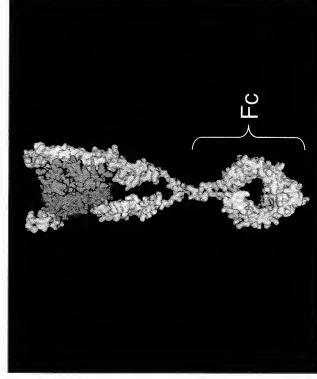
EXHIBIT A

Models of TNF Antagonists bound to TNF



Anti-TNF antibody

adalimumab
infliximab



Soluble TNF receptor

etanercept

TNF antagonists are shown in white, and TNF trimers are blue, green, and red. The TNF-binding site of the anti-TNF antibody is shown in cyan.

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EXHIBIT B

Fourth Edition

Immunology Immunopathology and Immunity

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Department of Pathology and Laboratory Medicine
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#692



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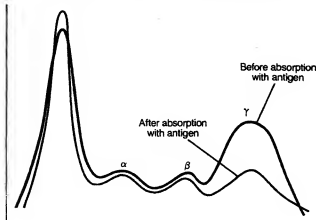
6 Antibodies, Immunoglobulins, and Receptors

Antibodies belong to a group of structurally related glycoprotein molecules found in the blood and extracellular fluids and known collectively as immunoglobulins. Immunoglobulins are the products of plasma cells, which secrete these proteins into serum and tissue fluids. Each plasma cell synthesizes and secretes large numbers of a single antibody that has the same antigen-binding specificity. Whereas some immunoglobulins are produced at all times in most normal animals, specific antibodies are a unique subset of immunoglobulins produced in response to antigenic stimulation. Given the enormous number of antigen specificities (epitopes) identifiable, an individual must have the ability to produce a great variety of antibody molecules. Cell surface antibodies on B cells serve as specific receptors for antigen; T cells also bear receptor molecules similar to antibodies, but having different structural components.

Gamma Globulin

Figure 6-1. If serum is placed under an electric gradient, the proteins will migrate in the charged field produced. The solid line depicts the serum protein electrophoresis pattern produced after absorption of serum from a hyperimmunized animal with the immunizing antigen. The dotted line depicts the protein pattern before absorption. It was thus shown that antibodies are largely found in the gamma globulins (the least negatively charged serum proteins).

The first identification of antibodies among the serum proteins was accomplished by electrophoresis in 1938 (Fig. 6-1). It was



found that antibodies are part of the gamma globulin fraction of serum. With further characterization of antibody molecules, this class of proteins has been shown to be very heterogeneous and the term *immunoglobulin* has been applied to designate this group of serum proteins. Immunoglobulins possess a degree of structural heterogeneity not found in most other serum proteins, but at the same time immunoglobulins also have structural similarities.

Myeloma Proteins






The study of the structure, synthesis, and function of human immunoglobulins has been made possible by the production of homogeneous immunoglobulins by plasma cell neoplasms (multiple myeloma, macroglobulinemia). From the sera of individuals with such tumors, homogeneous proteins can be isolated. These homogeneous immunoglobulins (myeloma proteins) can then be studied and structural analysis made that is not possible using normal immunoglobulins because of the great heterogeneity of normal immunoglobulins. The clinical features of multiple myeloma are presented in Chapter 28.

Immunoglobulins

Classes (Isotypes)

Five major immunoglobulin classes have been identified in man. Some of the characteristics of these immunoglobulins are given in Table 6-1. The five classes include immunoglobulins G (IgG), A (IgA), M (IgM), D (IgD), and E (IgE). The basic structural unit of each immunoglobulin class consists of two pairs of polypeptide chains joined by disulfide bonds (Fig. 6-2). The disulfide bonds may be reduced by mercaptoethanol. In the presence of denaturing agent (e.g., acid, urea) four polypeptide chains, two L (light)- and two H (heavy)-chains, are liberated. Each antibody molecule contains two identical light chains and two identical heavy chains. The intact molecule may be digested by proteolytic enzymes to yield other fragments (Fc and Fab fragments; Fig. 6-3). The term *Fab* is used because it is this fragment that binds antigen. *Fc* was applied to a non-antibody-binding fragment of rabbit antibody that crystallized in the test tube. The Fc fragments of most antibodies do not crystallize. The L-chains are shared by immunoglobulins of the different classes and can be divided into two subclasses, kappa (κ) and lambda (λ), on the basis of their structures and amino acid sequences. A given immunoglobulin molecule is either type κ or type λ . Approximately 60% of the serum immunoglobulin molecules contain κ -type L-chains, and 40% λ -type L-chains. The H-chains are unique for each immunoglobulin class and are designated by the Greek letter corresponding to the capital letter designation of the immunoglobulin class (α -chains for the H-chains of IgA, γ -chains for the H-chains of IgG). IgM and IgA have a third chain component, the J-chain, which joins the monomeric units.

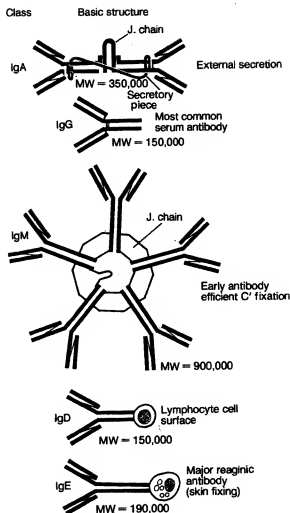
Table 6-1. Some Properties of Human Immunoglobulins

| Property | Immunoglobulin Class | | | | |
|---------------------------------|--|---|---|---|---|
| | IgG | IgA | IgM | IgD | IgE |
| |  |  |  |  |  |
| Serum concentration (g/100 ml.) | 12 | 0.4 | 0.12 | 0.003 | < 0.0005 |
| Sedimentation coefficient (S) | 7 | 7 (6.1, 13)* | 19 (24, 32)* | 7 | 8 |
| Molecular weight | 140,000 | 160,000 ^Δ | 900,000 | 180,000 | 200,000 |
| Electrophoretic mobility | γ | Slow β | Between γ and β | Between γ and β | Slow β |
| H-chains | γ | α | μ | δ | ϵ |
| L-chains | λ or κ | λ or κ | λ or κ | λ or κ | λ or κ |
| Complement fixation | Yes | No | Yes | No | No |
| Placental transfer | Yes | No | No | No | No |
| Percent intravascular | 40 | 40 | 70 | — | — |
| Half-life (days) | 23 | 6 | 5 | 3 | 2.5 |
| Percent carbohydrate | 3 | 10 | 10 | 13 | 10 |
| Antibody activity | Most Ab to infections; minor part of secondary response; Rh, hemagglutinins, LE factor | Present in external secretions | First Ab formed; ABO isoenzymes; rheumatoid factor | Antibody activity demonstrated, found on lymphocyte surface | Reagin sensitizes mast cells for anaphylaxis |

* Figures in parentheses indicates the existence of other molecular forms, such as polymers.

^Δ Serum IgA 160,000 MW; secretory IgA 350,000 MW, may activate alternate pathway (see Chap. 10) (Modified from Fahey, J.L.: J.A.M.A. 194:183, 1966).

Figure 6-2. Human immunoglobulin classes. Human humoral (circulating) antibodies belong to five classes: IgA, IgG, IgM, IgD, and IgE. The basic unit of each immunoglobulin molecule consists of two pairs of polypeptide chains joined by disulfide bonds. All immunoglobulins have the same L (light)-chain components, identifiable antigenically as kappa (κ) or lambda (λ), with any given immunoglobulin molecule having two κ -chains or two λ -chains. No naturally occurring immunoglobulin molecule has one κ -chain and one λ -chain. H (heavy)-chains of each immunoglobulin class are unique for that class and determine its biologic properties. H-chains of each immunoglobulin class are designated by the Greek letter corresponding to the capital letter identifying the class.



Biological Properties of Immunoglobulins

The five classes of immunoglobulins have different biological properties and are distributed differently in the intact animal. The structure responsible for the biological properties of each immunoglobulin class is located on that part of the immunoglobulin molecule that is unique for each class (the Fc portion of the H-chain).

Each IgG molecule consists of one H_2L_2 unit with a molecular weight of about 140,000. Molecules of the IgG class are actively transported across the placenta and provide passive immunity to the newborn infant at a time when the infant's immune mechanisms are not developed. IgG is widely distributed in the tissue fluids and is about equally divided between the intravascular and extravascular spaces.

IgM is the first immunoglobulin class produced by the

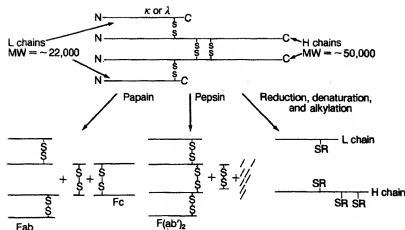


Figure 6-3. Human immunoglobulin fragments. The intact IgG molecule may be fragmented by different reagents into subunits. Digestion with papain occurs on the amino side of the interchain disulfide bond and results in three major fragments, two Fab and one Fc, and a minor fragment. Fab fragments consist of an L-chain and the amino half of an H-chain joined by a disulfide bond. The Fc fragment consists of the carboxy halves of H-chains joined by a disulfide bond. An additional small peptide from the middle of the heavy chains containing a disulfide bond

is also produced. The Fab fragment contains an antigen-binding site and reacts with, but does not precipitate, antigen because it is monovalent. The Fc portion is responsible for biological properties such as complement fixation. Digestion with pepsin occurs on the carboxy side of the interchain disulfide bond and results in two $F(ab')$ fragments joined by a disulfide bond because one of the disulfide bonds joining the H-chains is preserved. This fragment, $F(ab')_2$, reacts with and precipitates antigen because it is divalent (contains two antigen-binding sites).

Additional peptide fragments, some containing disulfide bonds, are produced by the action of pepsin, presumably due to further digestion of the Fc fragment. Reduction of disulfide bonds, alkylation of free SH groups ($R = CH_3CONH_2$), and denaturation of ionic and hydrogen bonds result in liberation of polypeptide chains — two L-chains (MW 22,000) and two H-chains (MW 50,000). Each polypeptide chain contributes to the antigen-binding site of the intact Fab fragment. That portion of H-chain present in the Fab fragment is called the Fd piece.

maturing fetus and may be the first immunoglobulin class representing a given antibody specificity following immunization (primary response). IgM occurs as five H_2L_2 units joined to each other by disulfide bonds located on the Fc part of the molecule and to the J-chain; its molecular weight is 900,000. IgM is found mainly in the intravascular fluids (80%). It is also the most efficient class of immunoglobulin in fixing complement and therefore is highly active in cytotoxic and cytolytic reactions (see Chapter 15).

IgM does not normally cross the placenta from mother to fetus, but may be produced actively by the fetus prior to birth, especially if the fetus has been exposed to antigens by infection. Thus IgM antibodies in the cord blood of the fetus are evidence of fetal immunization by exposure to infectious agents.

IgA is found in relatively small amounts in serum and tissue fluids, but is present in high concentrations in external secretions such as colostrum, saliva, tears, and intestinal and bronchial secretions. The IgA molecules in these fluids exist as dimers (two H_2L_2 units) joined by a J-chain and bound to an extra protein (*transport piece*). This transport piece is produced by secretory mucosal or glandular cells and facilitates the secretion of the dimeric IgA into the external fluids. Because IgA antibodies are prominent in external secretions, such antibodies are part of the first line of defense against infectious agents.

IgE is present in very low concentrations in serum and tissue fluids, but binds to a specific cell surface receptor on tissue mast cells (see Chapter 18). These cells are so named because they contain cytoplasmic granules and appear to have eaten (German *Mast*, "forced fattening"). Mast cells are armed by IgE antibodies that are bound to their surface receptors. Each antigen to which an individual is allergic may interact with cell-bound IgE and trigger the release of the granules. This releases biologically active molecules, such as histamine and serotonin. Antibody with this biological property is termed *reaginic antibody* or *reagin*.

IgD is present in very low concentrations in the serum. IgD is found on the surface of a high proportion of immature human B lymphocytes, suggesting that IgD may serve as a cellular receptor for antigen. The same variable region is used for IgD on the cell surface and for the IgM, IgG, or IgA that will ultimately be secreted. Thus, when antigen binds the IgD receptor, it stimulates the cell to multiply and ultimately to differentiate and to secrete antibodies of other classes that will be specific for the antigen.

Subclasses (Isotypes)

In addition to the five major classes of immunoglobulins in humans, subclasses of IgG, IgA, and IgM have been recognized. For example, four subclasses of IgG may be identified. These subclasses are designated IgG_1 , IgG_2 , IgG_3 , and IgG_4 . The subclasses differ in the sequence of their heavy chain constant regions (Table 6-2). IgG_1 molecules predominate in normal serum (9 mg/ml). The serum content of IgG_2 is 2.5 mg/ml, and

Table 6-2. Biological Properties of IgG Subclasses

| Property | IgG_1 | IgG_2 | IgG_3 | IgG_4 |
|----------------------------------|---------|--------------------------|---------|------------------|
| Percentage of total IgG in serum | 65 | 23 | 8 | 4 |
| Complement fixation | ++ | + | +++ | 0 |
| Placental transfer | +++ | ++ | +++ | +++ |
| Passive cutaneous anaphylaxis* | +++ | 0 | +++ | +++ |
| Receptor for macrophage | +++ | 0 | +++ | 0 |
| Reaction with staph protein A | +++ | +++ | 0 | +++ |
| Prominent antibody activity | Anti-Rh | Anti-levan, anti-dextran | Anti-Rh | Anti-factor VIII |

* Heterocytophilic antibody (see Chapter 18).

the serum content of IgG₃ and IgG₄ is 0.5 to 1.0 mg/ml. The biological significance of these immunoglobulin subclasses is not well understood. However, IgG₁ and IgG₃ are more active in fixing complement, whereas IgG₄ does not fix complement. IgG₂ predominates in the response to polysaccharide antigens and does not cross the placenta with the same efficiency as the other IgG subclasses. Therefore, the different IgG subclasses have different biological properties. In addition, the locations of the interchain disulfide bonds are different. IgA₁ and IgA₂ subclasses differ in sensitivity to bacterial proteases, and α_2 H and L chains are not joined to each other by disulfide bonds, as are α_1 chains, but are held together by electrostatic forces.

Antibodies

Primary Structure

The primary structure of a protein molecule is the sequence of amino acids that make up the light and heavy polypeptide chains (see Fig. 6-4). On the basis of antibody sequence data,

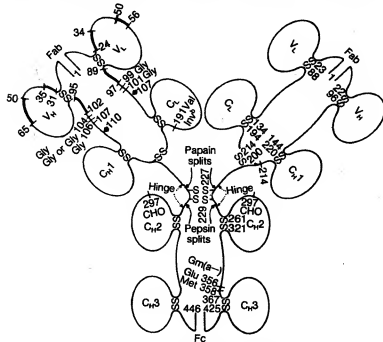


Figure 6-4. Schematic view of four-chain structure of human IgG molecule. Numbers on right side: actual residues of myeloma protein EU. Numbers of Fab fragments on left side aligned for maximum homology; light chains numbered as by E.A. Kabat (J Immunol 125:961, 1980). Hypervariable regions, complementarity-determining regions

(CDR): heavier lines. V_L and V_H: light- and heavy-chain variable regions. C_{H1}, C_{H2}, and C_{H3}: domains of constant region of heavy chain. C_L: constant region of light chain. Hinge region in which two heavy chains are linked by disulfide bonds is indicated approximately. Attachment of carbohydrate is at residue 297. Arrows at residues 107 and 110 denote

transition from variable to constant regions. Sites of action of papain before the hinge region and of pepsin after the hinge region show why papain produces Fab monomers and pepsin produces F(ab)₂ dimers. Locations of a number of heritable allotypic differences (Gm, Inv) are given.

EXHIBIT C

Soluble Tumor Necrosis Factor (TNF) Receptors Are Effective Therapeutic Agents in Lethal Endotoxemia and Function Simultaneously as Both TNF Carriers and TNF Antagonists

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ABSTRACT. Two forms (monomeric or dimeric) of the extracellular, ligand-binding portion of the human p80 cell-surface receptor for TNF were used to antagonize TNF activity in vitro and in vivo. The dimeric sTNFR:Fc molecule was a more potent inhibitor of TNF than the monomeric sTNFR (50 to 1000×), as assessed in vitro by inhibition of TNF binding or bioactivity and in vivo by protection of mice from an otherwise lethal injection of LPS. Surprisingly, the dimeric sTNFR:Fc construct demonstrated a beneficial effect even when administered 3 h after a lethal LPS injection (i.e., after serum TNF levels had peaked and receded). To study the mechanism by which the soluble TNFR functions in vivo, serum TNF levels were examined in mice given LPS in the presence or absence of soluble receptor. Administration of a mortality-reducing dose of sTNFR:Fc ablated the rise in serum TNF bioactivity that normally occurs in response to LPS. However, TNF bioactivity was revealed in these "TNF-negative" serum samples when the L929 bioassay was modified by inclusion of a mAb that blocks the binding of murine TNF to the human soluble TNFR. These results indicate that the absence of direct cytolytic activity in the L929 assay was caused by neutralization of TNF, rather than to an absence of TNF in the serum. Moreover, administration of either monomeric sTNFR or low doses of dimeric sTNFR:Fc actually resulted in increased serum TNF levels compared to mice given LPS but no soluble receptor. However, these "agonistic" doses of soluble receptor did not lead to increased mortality when an LD₅₀ dose of LPS was given. Thus, dimeric sTNFR are effective inhibitors of TNF and under some circumstances function simultaneously as both TNF "carriers" and antagonists of TNF biologic activity. *Journal of Immunology*, 1993, 151: 1548.

TNF is a polypeptide hormone released by activated macrophages and T cells, which mediates a wide range of biologic functions. In addition to its potential role as a regulator of the normal immune response, TNF is also thought to play a major role in systemic toxicity associated with sepsis (1-6). TNF may also be involved in the pathogenesis of AIDS (7-9) as well as a number of autoimmune and inflammatory diseases (10-13). A mole-

cule that specifically inhibits the biologic activities of TNF may thus have considerable therapeutic utility.

Soluble, extracellular, ligand-binding portions of cytokine receptors occur naturally in body fluids and are believed to regulate the biologic activities of cytokines (14-17). The importance of these molecules as cytokine regulators is underscored by the fact that several pox viruses encode proteins with structural and functional homology to the extracellular portions of the receptors for TNF and IL-1 (18-20). Considerable controversy exists concerning the type of regulatory role naturally occurring soluble cytokine receptors might perform. Although it is likely that such molecules will function as cytokine carriers in an operational sense by altering the biodistribution of the cytokine to which they bind, it is not clear whether such an interaction would serve to agonize or antagonize the biologic

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effects of the cytokine (21, 22). However, experiments in which recombinant soluble receptors have been administered *in vivo* demonstrate their potential to inhibit immune and inflammatory responses, presumably by acting as antagonists of cytokine activity (23, 24).

There are two distinct cell-surface receptors for TNF: the 80 kDa (p80) and the 60 kDa (p60) receptors, both of which bind TNF- α and TNF- β (25, 26). Given the predominantly trimeric nature of TNF (25) and the apparent requirement for cross-linking of cell-surface TNFR for signal transduction (27), it is likely that dimeric soluble receptor constructs should possess a higher affinity for TNF (28) and therefore function as considerably more potent competitive inhibitors than monomeric sTNFR.² This prediction has been verified by the results of recent experiments demonstrating superior TNF inhibitory activity of dimeric Fc fusion constructs of p60 *in vitro* (29). Although soluble forms of both monomeric and dimeric p60 TNFR have been shown to be beneficial in animal models of sepsis, no direct comparison of the *in vivo* potency of monomeric vs dimeric receptors in sepsis has been reported. In addition, little information is available concerning the mode of action of such inhibitors *in vivo*.

Monomeric and dimeric (Fc fusion protein) forms of the p80 TNFR were constructed and compared *in vitro* and *in vivo* for effects on TNF biologic activity. The results indicate that the sTNFR:Fc, but not the sTNFR, was effective in reducing mortality associated with LPS administration, at least over the concentration range tested. In addition, the sTNFR:Fc molecule can function simultaneously as both a TNF "carrier" and an antagonist of TNF biologic activity and thus inhibit the lethal effects of LPS by acting as a biologic buffer for TNF.

Materials and Methods

Mice

BALB/c female mice 8 to 10 wk old were purchased from Charles River (Wilmington, MA) and were maintained within a specific pathogen-free environment.

Construction and production of p80 sTNFR and sTNFR:Fc

Recombinant sTNFR was expressed in a CHO cell line using the glutamine synthetase selectable and amplifiable marker. For production, cells cultured to confluence in roller bottles were washed with PBS and then cultured in serum-free medium. Purification of the sTNFR from the CHO supernatant was accomplished in a single affinity

chromatography step using a mAb, M1, specific for sTNFR.

Recombinant sTNFR:Fc was expressed in CHO cells using the dihydrofolate reductase selectable and amplifiable marker. Suspension cells were centrifuged and resuspended into serum-free medium in a controlled bioreactor. The product was collected after 7 days. The sTNFR:Fc molecule was purified using protein A affinity chromatography followed by an ion-exchange step.

Concentrations of the purified sTNFR and sTNFR:Fc were determined by amino acid analysis. Endotoxin levels were determined to be <5.6 ng endotoxin/mg sTNFR or sTNFR:Fc using the Kinetic-QCL assay (Whittaker Bioproducts, Walkersville, MD) for detection of Gram-negative bacterial endotoxin. Physical characterization included SDS-PAGE, N-terminal sequencing, and immunoreactivity analyses (K. E. Stramler and H. Madani, unpublished observations). A diagrammatic representation of p80 sTNFR and sTNFR:Fc is shown in Figure 1.

Antibodies to soluble TNFR

The generation of mAb to the human p80 sTNFR has been described previously (30). M1 mAb (rat IgG 2b) and M3 (rat IgG) mAb both bind to the human p80 sTNFR but not to mouse TNFR.

Binding inhibition assay

Human rTNF- α was expressed in yeast as a protein composed of the entire coding region of mature TNF fused to an octapeptide at the N terminus, useful in affinity purification. Purified TNF was radiolabeled as described (18) to a sp. act. of 2×10^{15} cpm/mmol, without loss of biologic activity (measured in an L929 cytotoxicity assay) or receptor-binding activity (see below).

Inhibition assays were carried out as described (31). Briefly, [¹²⁵I]TNF- α (0.5 nM) was incubated in binding medium (RPMI 1640, 2.5% BSA, 50 mM HEPES buffer, pH 7.4, 0.4% NaN₃) for 2 h at 4°C with serially diluted inhibitors (human sTNFR:Fc, sTNFR monomer, or unlabeled human rTNF- α) and 2×10^6 U937 cells. Duplicate aliquots were subsequently removed, centrifuged through a phthalate oil mixture to separate free and bound ligand, and the radioactivity was measured on a gamma counter. Nonspecific binding values were determined by inclusion of a 200× molar excess of unlabeled TNF and were subtracted from total binding data to yield specific binding values. Data were plotted and results analyzed as described (31).

L929 bioassay for TNF activity

The protocol used to measure the presence of TNF cytotoxic activity using L929 cells as targets has been described previously (32, 33). Briefly, 10 μ l of mouse serum, mouse

² Abbreviations used in this paper: sTNFR, soluble monomeric human p80 TNFR; sTNFR:Fc, recombinant fusion protein composed of soluble dimeric human p80 TNFR linked to the Fc region of human IgG1; CHO, Chinese hamster ovary.

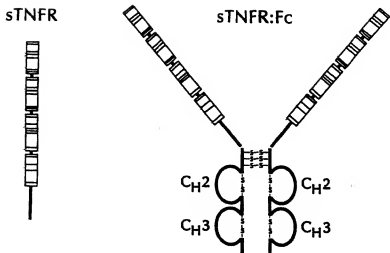


FIGURE 1. Construction of monomeric sTNFR and dimeric sTNFR:Fc molecules. Extracellular portions of the human p80 TNFR cDNA were cloned and produced as described in *Materials and Methods*. In the dimeric sTNFR:Fc molecule three disulfide bonds are depicted. However, the disulfide bond closest to the N terminus is normally used for binding to the Ig L chain and thus, its state (i.e., free cysteine or disulfide bond) in the sTNFR:Fc fusion product is not known.

rTNF- α (Genzyme, Boston, MA), or supernatant from LPS-stimulated RAW 264.7 cells (American Type Culture Collection, Rockville, MD) was serially diluted (50%:50%, v/v) in flat bottom, 96-well microtiter plates. L929 medium (RPMI 1640 with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin) was added to each well, followed by soluble receptors, control proteins, or mAb in a total volume of 30 μ l. Ten microliters of actinomycin D was then added (final concentration of 0.1 μ g/well; Sigma, St. Louis, MO). Finally, 5×10^4 L929 cells were added to each well (final volume/well = 100 μ l) and the plates were incubated at 37°C in 5% CO₂. To prevent the influence of edge effects on the TNF bioassay, only the inner wells of each plate were utilized. All outer wells received 200 μ l of L929 medium only. After 16 h of incubation, the culture medium was removed and replaced with 200 μ l of 0.5% crystal violet in methanol/water (1/4). The plate was washed with distilled water and air dried at ambient temperature. One hundred microliters of 2% deoxycholic acid (catalog no. D-6750, Sigma) was added to each well to solubilize the crystal violet and the plates were analyzed on an ELISA plate reader at 562 nm. The negative control consisted of L929 cells in the presence of actinomycin-D. Estimates of serum TNF concentrations were obtained by comparing the TNF activity in the experimental serum samples with the activity obtained with the mouse rTNF- α standard.

LPS-induced mortality

LPS, derived from *Escherichia coli* 0127:B8 (catalog no. DF3132-25, VWR, Seattle, WA), was resuspended at 10 mg/ml in sterile saline and stored at -20°C in small aliquots. The LPS was diluted to the proper concentration and sonicated (CU-6 sonicator; Branson, Shelton, CT) for 1 min before injection. BALB/c female mice (18 to 20 g) were injected i.v. with an LD₅₀ to LD₁₀₀ dose of LPS (300 to 400

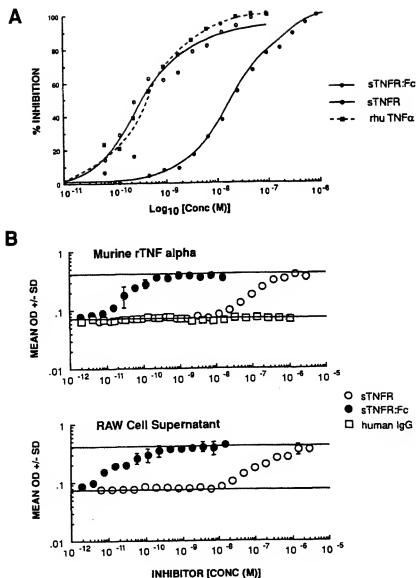
μ g) in 0.2 ml of saline. The LPS was injected either alone or in conjunction with sTNFR, sTNFR:Fc or control protein, human IgG (catalog no. I-4506, Sigma). In some experiments, mice were injected with LPS i.v. followed at 2, 3, or 4 h with an i.v. injection of soluble receptor or control protein. Survival was monitored for at least 5 days and, in some experiments, the mice were observed for a maximum of 4 wk. However, no further mortality occurred after the initial 5-day observation period.

Results

In vitro neutralization of TNF activity by soluble TNFR

The ligand binding characteristics of sTNFR monomer and sTNFR:Fc were determined by cell-based inhibition studies using ¹²⁵I-human rTNF- α and U937 cells expressing surface p80 and p60 TNFR. Results of these experiments are shown in Figure 2A. To generate a robust criterion of the relative activity of the sTNFR:Fc, we have analyzed the binding inhibition data with a simple one-site model to yield a single K_i , which reflects that concentration of inhibitor which mediates 50% inhibition of binding of TNF to cell-surface receptors. As predicted from (1) the multivalent interactions that occur between TNF ligands and receptors and (2) previous studies (29), the sTNFR:Fc ($K_i = 1 \times 10^{10}$ M⁻¹) shows ~50-fold higher affinity for the ligand than does the sTNFR monomer ($K_i = 2 \times 10^8$ M⁻¹). Thus, one might suspect that the sTNFR:Fc molecule would be a better antagonist of TNF biologic activity in comparison to the monomeric sTNFR in vitro and in vivo. To address the biologic efficacy of monomeric (sTNFR) and dimeric (sTNFR:Fc) forms of the soluble p80 TNFR, both molecules were analyzed for their ability to neutralize TNF activity in vitro in the L929 bioassay (Fig. 2B). Monomeric sTNFR and dimeric sTNFR:Fc inhibited the ac-

FIGURE 2. Comparison of TNF binding and neutralizing capability of sTNFR and sTNFR:Fc. **A**, U937 cells (2×10^6) were incubated at 4°C for 4 h with 0.5 nM [125 I]-human rTNF- α in binding medium and varying concentrations of inhibitor (sTNFR:Fc, sTNFR monomer or unlabeled human rTNF- α) in a total volume of 150 μ l. Duplicate 70- μ l aliquots of the suspension were subsequently removed and microfuged through a phthalate oil mixture to separate free and bound ligand. Radioactivity was measured in a gamma counter and the data were analyzed according to a simple competitive inhibition model. **B**, a constant amount of murine rTNF- α (125 pg/ml) or natural TNF (derived from LPS-stimulated RAW cells, 1/200 dilution) was added to each well of an L929 cytotoxicity assay in the presence of varying amounts of inhibitors (sTNFR, sTNFR:Fc or human IgG). Details of the L929 cytotoxicity assay are provided in *Materials and Methods*. The OD of L929 cells in the absence of TNF is indicated by the upper solid line (mean OD approximately 0.45) and maximal lysis of L929 cells is indicated by the lower solid line (mean OD approximately 0.075).



tivity of mouse TNF (recombinant or natural) in a dose-dependent fashion; however, sTNFR:Fc was approximately 1000-fold more efficient than sTNFR. Identical results were obtained when human rTNF- α was utilized as the ligand (data not shown). Human IgG, used as a control protein, had no effect on TNF activity.

Ability of sTNFR to prevent mortality induced by LPS

We have also compared the biologic efficacy of sTNFR and sTNFR:Fc in vivo in a murine model of LPS-induced septic shock. Various doses of sTNFR:Fc or control protein (human IgG) were mixed with a lethal dose of *E. coli* LPS (400 μ g/mouse) and injected i.v. into 18- to 20-g BALB/c female mice. Survival was monitored for 5 days and the results are presented in Figure 3. Treatment of mice with LPS only or LPS and any dose of human IgG resulted in 0 to 10% long

term survival. In contrast, 90% of mice treated with LPS plus 100 μ g (1.95 nmol) of sTNFR:Fc survived. Beneficial effects of the sTNFR:Fc protein were also evident with doses as low as 10 μ g (0.2 nmol)/mouse. In similar studies we have been unable to demonstrate an effect of recombinant monomeric sTNFR on survival even when doses as high as 260 μ g (10.35 nmol) were administered (Fig. 4). However, based on the in vitro neutralizing capacity of the monomeric vs dimeric sTNFR (Fig. 2) and the dose of sTNFR:Fc required to effect survival in vivo (Fig. 3), monomeric sTNFR would be predicted to demonstrate efficacy at much higher doses (10 mg/mouse).

The ability of the sTNFR:Fc protein to provide protection when given at various times after LPS administration was also tested. Mice received a lethal dose of LPS (i.v.) followed 2, 3, or 4 h later by sTNFR:Fc (100 μ g/mouse). Two to three separate experiments were conducted for each

FIGURE 3. Administration of sTNFR:Fc prevents mortality of BALB/c mice injected with a lethal dose of LPS. Various doses of sTNFR:Fc or human IgG, as a control, were mixed with a lethal dose of LPS (400 μ g) and injected i.v. into BALB/c mice. Survival was monitored at least once a day for 5 days. In each of three separate experiments, mice treated with sTNFR:Fc at doses of 10 μ g or above demonstrated enhanced survival.

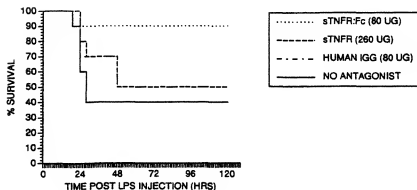
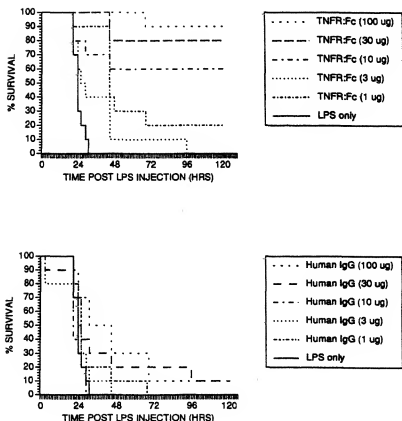


FIGURE 4. Administration of sTNFR does not affect mortality of BALB/c mice injected with a lethal dose of LPS. The procedure was identical to that described in the legend to Figure 3. Note: the response of mice treated with human IgG plus LPS overlaps the response of mice treated with sTNFR (260 μ g) plus LPS.

time point. All experiments provided similar results and therefore the results were pooled (Fig. 5). The results demonstrate that the administration of sTNFR:Fc was clearly beneficial even when administered up to 3 h after the injection of LPS. In the same experiment, the progression of serum TNF activity after LPS injection was determined in a subset of mice that received LPS only (Fig. 6). These experiments and previous reports (34–36) demonstrate that most of the serum TNF activity was produced during the first 2 h after LPS administration. These results demonstrate that the sTNFR:Fc protein was efficacious even when administered after serum TNF levels had peaked and receded. Thus, the efficacy of the sTNFR:Fc molecule must not be due solely to neutralization of serum TNF bioactivity.

Effect of sTNFR and sTNFR:Fc on serum TNF levels in vivo

To study the mechanism by which sTNFR:Fc protected mice from an otherwise lethal dose of LPS, the effect of the two forms of soluble TNFR on TNF activity present in the serum was examined. Mice were injected with LPS alone (400 μ g) or LPS mixed with 100 μ g of sTNFR, sTNFR:Fc, or control protein, human IgG. Serum samples were obtained 2 h after injection and assayed for TNF bioactivity (Fig. 7). Mice injected with LPS alone or LPS mixed with human IgG exhibited equivalent amounts of serum TNF activity (approximately 1 ng/ml) 2 h after LPS injection. In contrast, mice treated with LPS plus 100 μ g of sTNFR:Fc

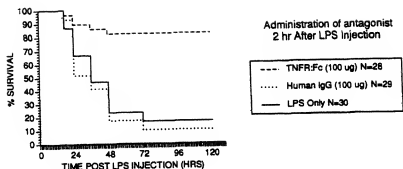
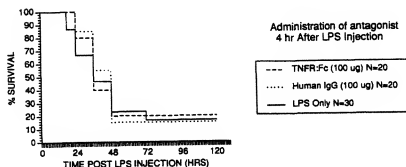
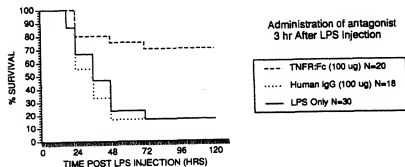


FIGURE 5. Administration of sTNFR:Fc prevents mortality of BALB/c mice even when injected 3 h after a lethal dose of LPS. At 2, 3, or 4 h after i.v. injection of a lethal dose of LPS (400 μ g), 100 μ g of sTNFR:Fc or human IgG, as a control, were injected i.v. Survival was monitored at least once a day for 5 days. The results represent a compilation of two to three separate experiments.



(which protects mice from the lethal effects of LPS injection, (Fig. 3)) had little or no serum TNF activity as assessed in the L929 assay. Somewhat surprisingly, mice treated with an equivalent dose of monomeric sTNFR (which was not efficacious in survival studies) exhibited serum TNF levels 10-fold higher (10 ng/ml) than control mice treated with LPS only or LPS plus human IgG.

Figure 8 depicts results of an experiment in which the relationship between the dose of sTNFR:Fc and serum TNF activity was examined. Sera obtained from mice injected with LPS alone or LPS plus 1 to 100 μ g of human IgG contained detectable TNF activity that titrated in a predictable fashion. Sera obtained from mice 2 h after treatment with 100 or 30 μ g of sTNFR:Fc and LPS contained little if any demonstrable TNF activity. Mice injected with 10, 3, or 1 μ g of sTNFR:Fc and LPS exhibited serum TNF activity but the sera displayed unusual characteristics. These serum samples demonstrated intermediate levels of

TNF activity, which failed to decrease even when diluted to 1/160 (Fig. 8) (data not shown). Because these results were obtained only when mice received LPS and low doses of the sTNFR:Fc, we examined the influence of the sTNFR:Fc on TNF activity in these samples.

Ability of sTNFR:Fc molecules to act as carriers of TNF

Experiments were conducted to determine the effect of blocking the TNF-binding ability of sTNFR:Fc molecules in vitro in the L929 cytotoxicity assay. To this end, we utilized a mAb (M1) that binds to the sTNFR:Fc molecule and blocks the ability of the soluble human TNFR:Fc protein to bind TNF. Another rat mAb (M3) that binds the sTNFR:Fc molecule but does not block TNF binding was used as a control. To examine the ability of M1 to block TNF binding to sTNFR:Fc proteins, constant amounts of sTNFR:Fc (200

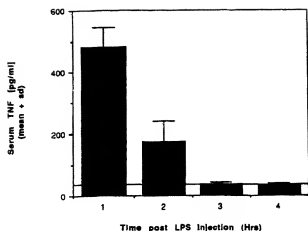


FIGURE 6. Serum TNF levels are elevated for 2 h after lethal LPS injection. Mice were injected with a lethal dose of LPS (400 μ g) and serum was obtained at 1, 2, 3, or 4 h. TNF activity was assessed by the L929 cytotoxicity assay as described in *Materials and Methods*.

ng/ml) and murine rTNF α (125 pg/ml) were added to dilutions of M1, M3, or rat IgG (Fig. 9). The ability of the sTNFR:Fc protein to neutralize the activity of TNF was reversed only in the presence of M1. In addition, full neutralization of the sTNFR:Fc protein (200 ng/ml) required a 10-fold excess (2 μ g/ml) of M1.

The effect of addition of M1 to serum obtained from mice 2 h after injection of LPS (400 μ g) mixed with 10 or 100 μ g of sTNFR:Fc was examined. As previously described (Fig. 8), the serum obtained from mice treated with 10 μ g of sTNFR:Fc demonstrated intermediate levels of activity that were not altered by dilution (Fig. 10). Addition of M1 (2 μ g/ml) to dilutions of the serum revealed the presence of additional TNF activity, which titrated in a predictable fashion. As expected, addition of control antibody (i.e., M3 or rat IgG) had no effect on the TNF activity. Furthermore, addition of M1, M3, or rat IgG had no effect on serum samples that did not contain the soluble human TNFR:Fc protein (i.e., sera obtained from mice injected with LPS and human IgG), demonstrating that the antibody did not affect the ability of mouse TNF to bind to the indicator L929 cells (Fig. 10). We have also examined serum samples from mice treated with a higher dose of sTNFR:Fc (100 μ g) and LPS. In the absence of manipulation these samples did not demonstrate TNF activity *in vitro*. However, TNF activity was revealed when serum from these mice was treated with M1 but not with M3 or rat IgG (Fig. 10). In fact, maximal activity in the L929 assay of the sera from mice injected with sTNFR:Fc (100 μ g) and LPS was still apparent at serum dilutions of 1/100, whereas sera obtained from mice treated with LPS only or LPS plus human IgG demonstrated only small amounts of TNF activity at a dilution of 1/16 (Fig. 10).

To determine whether or not sTNFR:Fc could prolong the presence of serum TNF, mice were injected with LPS and 10 or 100 μ g of sTNFR:Fc or human IgG, as described above, and serum samples were obtained at 4 h. The serum samples were assayed in the L929 bioassay in the presence and absence of M1, M3, or rat IgG (Fig. 11). As expected, sera obtained from mice injected 4 h previously with LPS alone or LPS plus human IgG did not contain serum TNF activity. However, sera obtained from mice injected with LPS plus sTNFR:Fc (10 or 100 μ g) still contained biologically active TNF, which titrated in a predictable fashion in the presence of M1 mAb. Thus, mice injected with LPS and the soluble human TNFR:Fc protein, even at therapeutic doses, retained increased levels of TNF in the serum that persisted for longer periods of time. However, depending upon the dose of sTNFR:Fc administered, the TNF activity was either (1) enhanced or (2) revealed only upon the addition of a mAb which blocked the binding of TNF to the sTNFR:Fc protein. These observations indicate that the binding of the sTNFR:Fc protein to TNF is reversible and that the inhibition of TNF activity reflects a balance between the presence of sTNFR:Fc, TNF, and endogenous TNFR (either cell surface or soluble).

The carrier function of sTNFR:Fc molecules is not detrimental to the host

As the administration of sTNFR:Fc under some circumstances produced increased levels of serum TNF (Fig. 8) that persisted for at least 4 h (Fig. 11), it was important to determine whether or not the administration of sTNFR:Fc molecules under these circumstances would lead to detrimental consequences. Mice were injected with a dose of LPS (300 μ g) which produced intermediate levels of mortality (60 to 70%), such that beneficial or deleterious effects of the TNFR could be observed. Mice treated with sTNFR:Fc at doses ranging from 10 ng to 10 μ g demonstrated equivalent or slightly better survival when compared with mice treated with LPS alone or LPS and human IgG (Fig. 12). Further experiments in which lower doses of sTNFR:Fc (100 pg to 1 μ g) were utilized yielded similar results (data not shown). Thus, administration of sTNFR:Fc in sublethal models of LPS toxicity had no detrimental consequences on the survival incidence.

Discussion

The data presented in this report demonstrate that a fusion molecule consisting of a soluble form of the extracellular portion of the p80 cell surface TNFR fused to the Fc portion of human IgG1 (sTNFR:Fc) is an effective antagonist of LPS-induced septic shock. An increased incidence of survival in mice given an otherwise lethal dose of LPS was observed when the sTNFR:Fc protein was injected 0 to 3

FIGURE 7. The effect of sTNFR vs sTNFR:Fc on serum TNF levels after co-administration of LPS in vivo. LPS (400 μ g) was mixed with 100 μ g of sTNFR, sTNFR:Fc, or human IgG and administered i.v. to BALB/c mice. Serum samples were obtained 2 h after injection and analyzed for TNF activity in the L929 cytotoxicity assay. The results were obtained from three to four separate experiments for each treatment group. The sensitivity of the TNF bioassay is approximately 50 pg/ml and is indicated by the solid line.

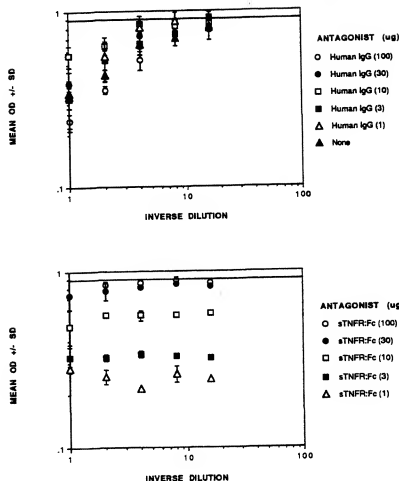
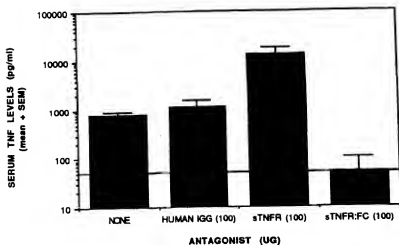


FIGURE 8. Analysis of TNF bioactivity in serum samples obtained 2 h after in vivo co-injection of LPS and sTNFR:Fc. A lethal dose of LPS (400 μ g) was mixed with varying doses of sTNFR:Fc or human IgG and injected i.v. into BALB/c mice. Serum was obtained from three mice in each group 2 h after injection. The serum for each group was pooled and analyzed for TNF activity in the L929 assay.

h after LPS administration (Figs. 3 and 5). When administered simultaneously with LPS, doses of sTNFR:Fc as low as 10 μ g (0.2 nmol)/mouse were beneficial (Fig. 3). In contrast, administration of up to 260 μ g (10.35 nmol) of the monomeric sTNFR failed to affect the incidence of mortality induced by LPS, even when the incidence of mortality in the control group was only 50% (Fig. 4). This difference

in efficacy between sTNFR:Fc and sTNFR in vivo may be explained in large part by the higher affinity of TNF for sTNFR:Fc than sTNFR, which results in a substantially greater ability of sTNFR:Fc to neutralize the biologic effects of TNF (Fig. 2). Furthermore, linkage of the sTNFR to the Fc region of Ig imparts a fivefold longer serum $t_{1/2}$ to the sTNFR:Fc molecule after i.v. injection (37), a property

FIGURE 9. Inhibition of the TNF neutralizing capacity of the human p80 sTNFR:Fc molecule by M1 but not M3 mAb. Dilutions of M1, M3, or rat IgG were added to constant amounts of sTNFR:Fc and murine rTNF- α in the L929 assay as described in *Materials and Methods*.

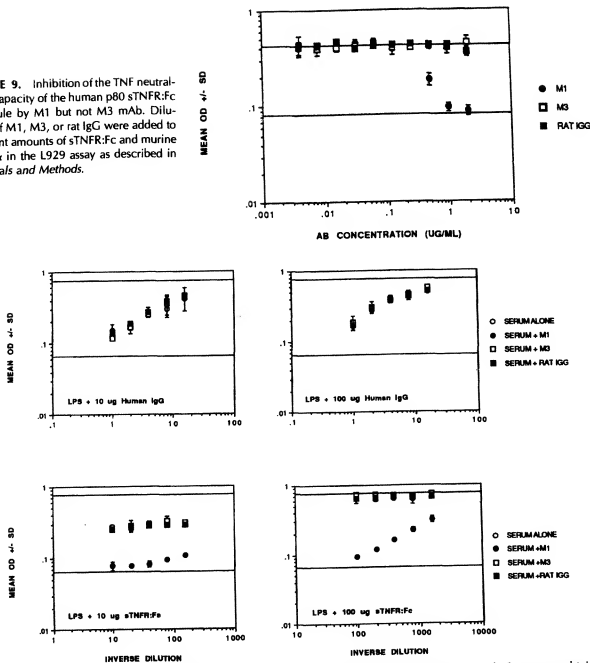


FIGURE 10. Demonstration of serum TNF activity *in vitro* in the L929 assay in the presence of M1 mAb. Serum was obtained from mice 2 h after injection of LPS (400 μ g) mixed with sTNFR:Fc or human IgG. The serum samples were serially diluted and assayed in the L929 cytotoxicity assay either alone or in the presence of constant amounts (2 μ g/ml) of M1, M3, or rat IgG.

that could also influence its efficacy *in vivo*.

The ability of sTNFR to alter the magnitude and time course of serum TNF after co-administration with LPS *in vivo* was examined. Sera from mice that received high, life-saving doses of sTNFR:Fc (e.g., 100 μ g) failed to exhibit significant levels of TNF bioactivity when assayed directly in the L929 cytotoxicity assay. However, further experimentation demonstrated that TNF was present in the serum but it was biologically inactive because of the con-

comitant presence of sTNFR:Fc. TNF activity in these samples was revealed in the presence of a mAb which blocked the ability of the human sTNFR:Fc molecules to bind TNF but did not interfere with the ability of TNF to bind to the murine TNFR on the surface of the L929 indicator cells (Figs. 9 to 11). These results suggest that the sTNFR:Fc protein has a relatively high exchange rate for TNF, such that once TNF is released *in vitro*, it can be detected if the TNF is inhibited from subsequently binding to free

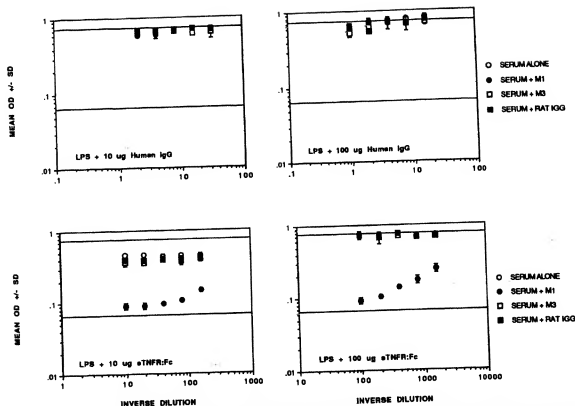


FIGURE 11. Prolongation of serum TNF in vivo by sTNFR:Fc. The protocol was identical to that described in the legend to Figure 10 except that the serum samples were obtained 4 h after LPS injection.

sTNFR:Fc molecules. If TNF is also released from the sTNFR:Fc molecule in vivo, the sTNFR:Fc molecules may function by dissipating the peak in serum TNF levels normally associated with bolus LPS injection.

Soluble TNFR:Fc molecules also function as carriers of TNF in that they alter the rate at which TNF disappears from the serum of LPS-treated mice. Control mice injected with LPS alone or LPS and human IgG had elevated serum TNF levels only during the first 2 h after injection (Figs. 6, 10, 11). However, mice treated with LPS and sTNFR:Fc retained TNF in their serum for at least 4 h (Fig. 11). In support of these data, we have demonstrated that the $t_{1/2\beta}$ of labeled TNF is increased approximately fourfold in vivo when injected concomitantly with sTNFR:Fc (D. Lynch and K. M. Mohler, unpublished observations). These results suggest that the sTNFR:Fc protein functions as an effective antagonist of LPS induced mortality by acting as a biologic buffer for TNF activity.

When mice were exposed to lethal doses of LPS and low doses of sTNFR, which failed to affect mortality incidence, serum TNF levels as detected in the L929 bioassay were elevated in comparison to control mice receiving LPS alone or LPS plus IgG (Figs. 7 and 8). However, despite the fact that low doses of sTNFR increased serum TNF activity, no agonistic activity in terms of mortality could be demonstrated when low doses of sTNFR were administered in

conjunction with an LD₅₀ dose of LPS (Fig. 12) (data not shown). These data indicate that the agonistic effects on serum TNF activity obtained in vivo in the presence of sTNFR were distinct from the effects of sTNFR on LPS-induced mortality. Alternatively, the sTNFR may function as an agonist only with lethal doses of LPS. If the latter hypothesis is correct, then lower (nonlethal) doses of LPS may induce sufficient quantities of endogenous soluble TNFR so that the administration of exogenous sTNFR:Fc molecules would have relatively minor additional biologic impact.

Several types of TNFR/antibody-based fusion proteins have been described and tested for efficacy in murine LPS-induced mortality models (38, 39). These TNF antagonists include the molecule employed in the present study, composed of the extracellular portion of the p80 cell-surface receptor linked to the Fc region of human IgG1, as well as molecules consisting of fusions between the extracellular portion of the p60 TNFR combined with the Fc region of either human IgG1 (38) or human IgG3 (39). The dose of p60 sTNFR:Fc (4 to 20 μ g) (38, 39) and the dose of p80 sTNFR:Fc (10 to 100 μ g) (Fig. 3) required to demonstrate efficacy are similar. However, efficacy of the different constructs was influenced substantially by the timing of administration relative to lethal LPS injection. The p80 sTNFR:Fc (human IgG1) construct was efficacious when

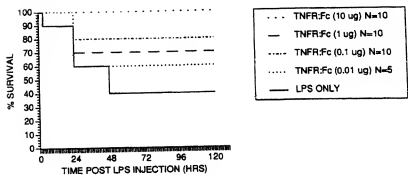
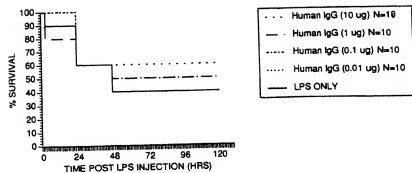


FIGURE 12. Administration of low doses of sTNFR:Fc is not detrimental to the host. BALB/c mice received an LD₅₀ dose of LPS (300 µg) premixed with low doses of sTNFR:Fc or human IgG. Survival was monitored at least once a day for 5 days.



administered as late as 3 h after LPS administration (Fig. 5). However, a sTNFR:Fc fusion protein consisting of the p60 sTNFR coupled to human IgG1 was effective only if administered within 1 h after lethal LPS injection (38). In contrast, preliminary reports utilizing the p60 sTNFR coupled with human IgG3 indicated that partial benefits were obtained as late as 3 h after LPS injection (39). Thus, significant differences exist between the published abilities of different sTNFR:Fc fusion proteins to function after LPS administration, and these differences do not appear to correlate with either the particular sTNFR (i.e., p60 or p80) or with the subclass of human IgG utilized for the fusion protein.

The relationship between serum TNF activity and efficacy of the sTNFR:Fc molecule had not been established before the present study. Given the ability of sTNFR:Fc to function effectively when administered as late as 3 h after LPS injection (Fig. 5), it was somewhat surprising to observe that the vast majority of detectable serum TNF activity had already passed by 3 h (Fig. 6). A number of hypotheses, which are not necessarily mutually exclusive, may explain these results. First, the length of time that TNF must be bound to its cell surface receptor prior to the induction of an irreversible biological effect such as cell lysis is unknown. However, studies by Engelberts et al. (40) suggest that TNF must be present for extended periods of time to achieve maximal biologic activity *in vitro*. Thus, sTNFR:Fc may be able to compete for TNF which has

already bound to the cell surface and, in effect, dislodge it before the interaction has occurred for a time sufficient to result in complete biologic signaling. In this regard, the rate of dissociation of radiolabeled TNF from its cell surface receptor *in vitro* is increased in the presence of either unlabeled TNF (41) or the dimeric sTNFR:Fc (C. Smith, unpublished results). Second, LPS-induced mortality may result from the cumulative effect of TNF. Thus, inhibition of the small amount of TNF present late in the time course might be sufficient to prevent mortality. Third, the therapeutic potential of the sTNFR:Fc molecule may not be related solely to the removal of serum TNF activity. The sTNFR:Fc molecule could function by inhibiting TNF activity in extravascular sites. Finally, LPS-induced toxicity may be mediated at least in part by TNF expressed on the cell surface, which may be masked in the presence of sTNFR:Fc. Regardless of the mechanism of efficacy of the sTNFR:Fc molecule, there is a relatively small window of time, 3 to 4 h after LPS injection, during which serum TNF levels are low and administration of the sTNFR:Fc molecule is still efficacious. These results also suggest that serum TNF levels may not always be a good prognostic indicator for the clinical efficacy of the sTNFR:Fc molecule.

Soluble TNF-binding proteins have been recovered from the urine of normal humans (42, 43) and appear at elevated levels in the serum of cancer patients (44, 45) and in response to endotoxin challenge (46). The biologic role of these TNF-binding proteins is currently under investigation.

tion. Previous investigators have demonstrated that TNF spontaneously loses activity in vitro and, under some circumstances, soluble p60 and p80 TNFR can prevent its spontaneous degradation, thereby enhancing the biological longevity of TNF (47). Our experiments demonstrated that a sTNFR monomer can function as an agonist of serum TNF activity in vivo and a sTNFR:Fc molecule could act either as an agonist or antagonist of serum TNF levels in a dose dependent fashion. Thus, the biologic effect of the soluble TNF-binding proteins isolated from humans will probably vary depending upon the relative concentration of TNF and sTNFR. This concept is supported by recent data of Girardin et al. (48), demonstrating increased concentrations of both TNF and soluble TNFR in the serum of septic patients. In that study, higher ratios of soluble TNFR to TNF correlated with increased probability of survival.

These experiments indicate that the sTNFR:Fc molecule is an effective antagonist of LPS-induced septic shock and are in agreement with a number of studies that have shown the beneficial effects of antagonizing TNF activity in sepsis with either antibody (1-3) or soluble receptors (38, 39). In aggregate these results indicate that TNF plays a central role in mediating the lethality associated with sepsis. However, several lines of evidence suggest that the role of cytokines in sepsis is not yet fully understood. First, antagonism of several cytokines other than TNF (e.g., IFN- γ and IL-1) can also lead to beneficial results (49, 50). Second, anti-TNF antibodies have been reported to have variable therapeutic potential in models of endotoxemia, cecal ligation and puncture and bacterial sepsis (51-53). Further experimentation will be required to determine whether or not the sTNFR:Fc molecule also displays the same spectrum of efficacy. However, the results presented here suggest that the sTNFR:Fc molecule may be a useful therapeutic agent for sepsis and other inflammatory diseases.

Acknowledgments

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EXHIBIT D

Monoclonal Antibodies to Human Tumor Necrosis Factors Alpha and Beta: Application for Affinity Purification, Immunoassays, and as Structural Probes

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ABSTRACT

Monoclonal antibodies were produced against two structurally related tumor necrosis factors (TNFs), TNF- α (previously called tumor necrosis factor) and TNF- β (previously called lymphotoxin). The potential of these antibodies for the purification of TNFs, the development of specific immunoassays, and for defining the antigenic and functional domains of these cytokines was investigated. None of the monoclonal antibodies cross-reacted with both TNF- α and TNF- β , or reacted with synthetic peptides which represented several of the regions of homology between these cytokines. Neutralizing monoclonal antibodies were utilized as immunoadsorbents to purify recombinant TNF- α and TNF- β from *E. coli* lysates. TNFs purified by this method were greater than 98 percent pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and exhibited specific activities that were the same as TNFs isolated from natural sources using conventional chromatographic techniques. In addition, specific ELISA assays were developed that could detect less than 1 ng/ml of TNF- α or TNF- β , and in contrast to bioassays, could discriminate between these related cytokines.

INTRODUCTION

Tumor necrosis factor (TNF) and lymphotoxin (LT) were originally described as activities that were cytostatic or cytotoxic for a number of tumor cell lines *in vitro*, but had no apparent harmful effects on normal cells in culture (1-8). These two factors had similar biological activities and were initially distinguished from each other solely on the basis of cellular source and induction conditions. Tumor necrosis factor was described as an activity that could cause the necrosis of transplanted tumors in mice (1-4). It was first discovered in the serum of mice and rabbits after they had been injected with bacillus Calmette-Guerin and endotoxin (1,4). TNF activity was also found in the media of activated mononuclear cell cultures (2-4), and was thought to be produced by macrophages (1-4). Lymphotoxin was the name given to a factor with anti-tumor cell activity that was found in the media of activated lymphocyte cultures (5-8). These factors were produced in very small amounts by normal cells, which made their purification and characterization

difficult. Recently, we have reported the purification of two cytotoxic factors derived from continuous tumor cell lines. TNF- α (previously called TNF) produced by a human promyelocytic leukemia cell line (HL60) and TNF- β (previously called LT), produced by a human B lymphoblastoid cell line (RPMI 1788) (9-11). Most of the amino acid sequences of both molecules were determined (10,11) and were found to be different but related. Using this amino acid sequence information, recombinant DNA techniques were utilized to clone the cDNAs for TNF- α and TNF- β and to express biologically active molecules in *E. coli* (12,13). A comparison of the complete amino acid sequences of these two proteins shows that 28 percent of the residues are identical and 51 percent are homologous, representing conservative amino acid changes (11,13). In addition, both cytokines have been shown to bind to the same cell surface receptor (14), and their genes have been found to be closely linked on chromosome six (15). Furthermore, it has been shown that both TNF- α and TNF- β can cause necrosis of Meth A tumors *in vivo* (12,13), a property which was originally used to name TNF. Due to these structural and functional similarities, TNF and LT have been renamed TNF- α and TNF- β (16,17), a nomenclature analogous to that used for interferons.

TNF- α and TNF- β cannot be distinguished on the basis of cytolytic activity in a standard L-929 cell lysis bioassay (1,6). In addition, the purification procedures used for TNF- α and TNF- β are complicated multi-column processes (9-11), making it cumbersome to purify TNFs from natural or recombinant sources. We, therefore, produced monoclonal antibodies against TNF- α and TNF- β in order to develop simple immunoaffinity purification procedures for these proteins, to discriminate between TNF- α and TNF- β by selective neutralization and quantitative immunoassays, and for use as structural probes to define the regions of the molecules responsible for their cytolytic activity.

In this report, we describe the isolation of both neutralizing and non-neutralizing monoclonal antibodies to TNF- α and TNF- β . These antibodies were used to purify recombinant TNFs, yielding homogenous biologically active proteins. Various monoclonal and polyclonal antibodies were used to develop specific ELISA assays that were about as sensitive as a standard L-929 cytolytic assay (11) but were faster and could discriminate between TNF- α and TNF- β . Binding studies and neutralization assays showed that none of the monoclonal antibodies reacted with both TNF- α and TNF- β . In addition, none of the antibodies showed any reactivity with synthetic peptides which represented some of the regions of homology between these cytokines. Several non-neutralizing antibodies were identified which reacted with a synthetic peptide that represents the amino-terminal region of TNF- β . These antibodies were also specific for a 171 amino acid form of TNF- β and did not react with a biologically active truncated form which lacks 23 N-terminal amino acids.

MATERIALS AND METHODS

Immunization Protocols

BALB/c mice were immunized with a combination of natural human TNF- α , purified from HL-60 cell line supernatants (11) and *E. coli* derived human recombinant TNF- α (13), purified using a similar protocol. Mice received an initial injection of natural TNF- α (1.7 μ g) emulsified in CFA and administered IP and SC. The animals were then boosted SC and IM at seven-day intervals with TNF- α adsorbed onto alum (0.1 ml of Al(OH)₃, 1.74 percent w/v in PBS), once with natural TNF- α (1.7 μ g) and once with recombinant TNF- α (10 μ g). Serum collected after the second boost was positive for neutralizing antibodies. Two weeks later, the animals were further boosted with recombinant TNF- α , 50 μ g injected IV and 125 μ g emulsified in IFA and injected SC. Nine weeks after the initial immunization and prior to fusion, the mouse which produced the highest titer of antibodies was injected with a large dose of recombinant TNF- α (1.4 mg) using a protocol that has been reported to

result in the production of high numbers of positive hybridomas (18) and yield high affinity antibodies (19).

BALB/c mice were immunized with TNF- α purified from RPMI 1788 cell line supernatants (9). The immunization protocol used for TNF- α was significantly different from that used for TNF- β . Several attempts to immunize mice with TNF- α yielded low titer antibodies that failed to neutralize biological activity. In an attempt to enhance its immunogenicity, TNF- α was polymerized by treatment with glutaraldehyde (1 M). Mice received an initial injection of polymerized TNF- α (poly-TNF- α) (50 μ g) emulsified in IFA and administered SC. Twice, at seven-day intervals, the animals were boosted IM and IP with poly-TNF- α (100 μ g) emulsified in IFA. Serum collected after the second boost was positive for antibodies by ELISA, but failed to neutralize biological activity. One week later, the mice were further boosted SC with poly-TNF- α (100 μ g) adsorbed on alum and IV with untreated TNF- α (10 μ g). Serum collected after the third boost was positive for neutralizing antibodies. Eight weeks after the initial immunization, the mouse that produced the highest titer antibodies was boosted IV with untreated TNF- β (15 μ g) and its splenocytes were harvested for fusion four days later.

Polyclonal antisera was obtained by immunizing NZW rabbits IO, at several sites, with recombinant TNF- α (1 mg) or TNF- β (1 mg) emulsified with CFA. At two-week intervals, the animals received booster injections SC, at several sites, with TNF- α (200 μ g) or TNF- β (200 μ g) emulsified with IFA. Ten days after booster injections, sera were collected and evaluated by ELISA and for neutralization of biological activity. The resulting pooled antisera had a neutralizing titer of approximately 900×10^3 units and 45×10^3 units of TNF- α and TNF- β neutralized per ml of serum, respectively.

Fusion and Cell Culture

Splenocytes were harvested from hyperimmune mice and were fused with NP3X63AG8.653 murine myeloma cells as previously described (20). The hybridomas were cultured in HAT medium (DMEM; glutamine, 2.9 g/l; 2-mercaptoethanol, 50 μ M; aminopterin, 10 μ M; hypoxanthine, 100 μ M; thymidine, 31.4 μ M supplemented with 10 percent NCTC 135 medium and 20 percent FBS), in five 96-well tissue culture plates which contained 2×10^4 BALB/c peritoneal macrophage feeder cells/well. The hybridomas were selected in HAT medium for fourteen days, and grown in HT medium (same as HAT but lacking aminopterin) thereafter. The cultures were screened by ELISA about fourteen days after fusion and positive cultures were cloned by limiting dilution in 96 well plates, using BALB/c thymocytes (10^6 /well) as feeder cells.

ELISA Screening Assay

Micro-ELISA plates (Dynatech Immulon II round bottom) were coated for 16 hours at 4°C with purified recombinant TNF- α (170 ng/well) or recombinant TNF- β (100 ng/well) in 50 mM sodium carbonate buffer, pH 9.6. The unreacted protein binding sites on the wells were blocked by incubating for 30 minutes at 22°C with 1 percent gelatin (w/v) in 150 mM NaCl, 50 mM Tris, 2 mM EDTA pH 7.4 (TBS) (150 μ l/well). The plates were then washed once with PBS containing 0.05 percent Tween 20 (PBS-Tween). Hybridoma culture supernatants serially diluted with TBS containing 5 mg/ml BSA and 0.05 percent Tween 20 (sample buffer), were added to antigen coated wells and allowed to bind for 2 hours at 22°C. After five rinses with PBS-Tween, horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel) (100 μ l/well) diluted 1:10,000 with sample buffer was added and allowed to bind for 1 hour at 22°C. The plates were rinsed five times with PBS-Tween and incubated 30 minutes at 22°C with 0.1 mg/ml o-phenylenediamine in 0.1 M phosphate-citrate buffer, pH 5.0 containing 0.012 percent H_2O_2 (100 μ l/well). The reaction was stopped by the addition of 2.5 N sulfuric acid (50 μ l/well) and the absorbance of each well was determined using a Titertek Multiscan autoreader. The titers were expressed as the reciprocal of the dilution required to achieve 50 percent of the maximum absorbance at 492 nm.

Neutralization of TNF Cytotoxicity

Supernatants derived from growing hybridomas or purified monoclonal antibodies were serially diluted with RPMI 1640, 50 U/ml penicillin, and 50 µg/ml streptomycin supplemented with 10 percent FBS. These test samples were incubated with an equal volume of the same medium containing approximately 200 U TNF- α or TNF- β for 16 hours at 37°C. The samples were then assayed for lysis of murine L-929 cells (11). These incubation conditions did not significantly reduce TNF- α or TNF- β biological activity in the absence of added antibody.

Monoclonal Antibody Isotyping

The isotype of the various anti-TNF monoclonal antibodies was determined by ELISA. Micro ELISA plates were coated for 16 hours at 4°C with isotype-specific rabbit antiserum (Miles) (100 µl/well diluted 1/1000 in 50 mM sodium carbonate buffer pH 9.6). Undiluted hybridoma culture supernatants were incubated in the coated wells for 2 hours at 22°C. The washing and further processing were done as described above for the ELISA screening assay.

Screening of Monoclonal Antibodies for Use in Immunoaffinity Chromatography by ELISA

The binding of anti-TNF monoclonal antibodies to antigen under various pH conditions and chaotropic salt concentrations was measured by ELISA. Test antibodies were allowed to bind to TNF- α or TNF- β coated micro ELISA wells for two hours at 22°C. Thereafter, the wells were washed five times with PBS containing 0.05 percent Tween 20. The wells were then treated for 1 hour at 22°C with either PBS, 0.15 M NaCl, 50 mM sodium acetate pH 4.0-7.0, 0.15 M NaCl, 50 mM sodium borate pH 7.5-11.0, 1.25 M KSCN, 20 mM tris pH 7.4; or 3.5 M KSCN, 20 mM tris pH 7.4. The wells were then washed five times with Tween 20 and further processed as described above for the ELISA screening assay. The absorbance values of treated wells were compared with PBS control wells and any signal less than 50 percent of the PBS well was considered to be negative for binding.

Purification of Monoclonal Antibodies

Hybridoma cells were grown as ascites tumors in pristane primed BALB/c mice as previously described (21). Monoclonal antibodies were then purified from ascites fluid by DEAE Affigel Blue (BioRad) chromatography (22). Monoclonal antibodies were also purified from tissue culture supernatants using a similar procedure. Hybridoma cells were cultured in a modified low serum medium containing: high glucose DMEM/F12 (1:1); Hepes, 10 mM; β -mercaptoethanol, 50 µM; ethanolamine, 20 µM; glutamine, 2.9 g/l; insulin, 5 mg/l and supplemented with 2.5 percent FBS (23). Cells were grown to a density of about 10^6 cells/ml in spinner flasks, the medium was harvested by filtration through a 3 µm filter (Pall) and concentrated 10-fold by ultrafiltration using a PM10 membrane (Amicon). The IgG fraction was isolated by precipitation with 50 percent saturated ammonium sulfate, and then dialyzed against 20 mM tris pH 7.2. Thereafter, the IgG was purified by DEAE Affigel Blue chromatography. Purified antibodies were concentrated about 10-fold by ultrafiltration using a PM10 membrane to a final concentration of about 10 mg/ml.

Preparation of Immunoabsorbent Sepharose

Purified monoclonal antibodies were extensively dialyzed against PBS and adjusted to a final concentration of 2 mg/ml. Cyanogen bromide activated Sepharose 4B (Pharmacia) was soaked in 1 mM HCl for 10 min and was then washed on a buchner funnel with 1 mM HCl (200 ml per gram dry gel). The washed gel

was rinsed with 1 gel volume of PBS and was mixed with the solution of purified monoclonal antibody at a ratio of 1 ml packed gel/ml antibody solution. The gel was allowed to react for 16 hours at 4°C with gentle agitation. The unreacted groups on the gel were blocked by incubating with an equal volume of 0.9 M ethanolamine-HCl, pH 8.0, for 2 hours at 4°C. The gel was then washed first with 5 volumes of PBS, and then with 5 volumes of 0.15 M NaCl, 0.1 M acetic acid and then with 10 volumes of 0.15 M NaCl, 0.05 M tris, 2 mM EDTA and stored in this buffer at 4°C.

Immunoaffinity Chromatography

All purification steps were carried out at 4°C. Frozen recombinant *E. coli* cells containing TNF- α or TNF- β expression plasmids (12,13) were suspended in 10 volumes of lysis buffer (0.2 M NaCl, 0.1 M tris, 50 mM EDTA pH 7.5) and then sonicated at full power for 10 min. at 50 percent duty cycle using a Branson W-375 sonicator. The extract was clarified by the addition of polyethyleneimine (pH 8.0) to a final concentration of 0.5 percent (w/v) and then centrifuged at 1100 x g for 15 min. The pellet was discarded, and solid ammonium sulfate was added to the supernatant to 50 percent saturation (TNF- α) or 40 percent saturation (TNF- β). After incubation for 16 hours at 4°C, the precipitate was collected by centrifugation at 10,000 x g for 15 min. The resulting pellet was dissolved in 1 volume (1 ml/gr. cells) of 0.1 M tris, 5 mM EDTA pH 7.5; and filtered using a 0.45 μ m millipore membrane filter. This sample was then loaded onto an immunoabsorbent column pre-equilibrated with TBS at a flow rate of 9.5 cm/hour. After washing the column with 10 volumes of TBS containing 0.05 percent Tween 20 and then with 1 volume TBS at a flow rate of 19 cm/hour, the TNFs were eluted with 0.15 M NaCl, 0.1 M sodium acetate pH 4.5 at a flow rate of 9.5 cm/hour. Fractions were collected into one-tenth volume of 1 M tris pH 8.5 to adjust the pH of the eluate to about 7.8. The columns were washed with about 10 volumes TBS prior to reuse.

Double Sandwich ELISA for TNF- α and TNF- β

Micro-ELISA wells (NUNC) were coated for 16 hours at 4°C with purified anti-TNF- α 1B2-G3 (100 ng/well) or anti-TNF- β 1G11-E4 (200 ng/well) diluted in PBS. Thereafter, the wells were washed once with PBS-Tween and samples, serially diluted with sample buffer, were added and incubated for 2 hours at 22°C. The wells were then washed 5 times with PBS-Tween and incubated for 2 hours at 22°C with rabbit anti-TNF- α or anti-TNF- β (100 μ l), diluted with sample buffer 1:1600 and 1:10,000, respectively. The wells were again washed 5 times with PBS-Tween and incubated for 1 hour at 22°C with goat anti-rabbit HRP conjugate (Cappel) (100 μ l), diluted 1:10,000 with sample buffer. The wells were then washed 5 times and incubated for 30 min. with 0.2 mg/ml o-phenylenediamine in 0.1 M phosphate/citrate pH 5.0 containing 0.012 percent H₂O₂ (100 μ l). The reaction was stopped by the addition of 2.5 N H₂SO₄ (50 μ l/well) and OD₄₉₂ was measured using a Titertek Multiscan autoreader interfaced with an HP computer with Titercalc^R software. Both assays had useful ranges of 0.4-25 ng TNF/ml and their specificity was demonstrated by the lack of a signal generated by 30 μ g/ml of TNF- α in the TNF- β ELISA or by 30 μ g/ml of TNF- β in the TNF- α ELISA.

Gel Electrophoresis and Immunoblots

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (24). Gels were stained with 0.15 percent Coomassie Blue R250 (w/v) in 25 percent isopropanol (v/v), 10 percent acetic acid (v/v), and destained with 10 percent methanol (v/v), 10 percent acetic acid (v/v). To determine purity, gels were analyzed using a scanning laser densitometer (LKB). The binding of antibodies to TNFs separated by SDS-PAGE was assessed by the immunoblot technique. Recombinant TNF- α (2 μ g) or 18 kDa TNF- β (2 μ g) were subjected to electrophoresis on 15 percent acrylamide

SDS-PAGE gels and transferred to nitrocellulose as previously described (25). The reactive sites on the nitrocellulose were blocked by incubating for 30 min with 3 percent gelatin (w/v) in TBS. Test monoclonal antibodies were diluted in 10 μ g/ml in 1 percent gelatin (w/v) in TBS and incubated with the nitrocellulose strips for 16 hours at 22°C. The strips were then washed for 15 min each with three changes of PBS-Tween. Bound monoclonal antibodies were detected by incubating for 4 hours with HRP-conjugated anti-murine IgG (Cappel) diluted 1:1000 in 1 percent gelatin. The strips were washed as above and incubated 15 min with 4-chloro-1 naphthol (0.6 mg/ml in 20 percent methanol, 80 percent TBS containing 0.018 percent H_2O_2), rinsed with water and air dried.

Protein Determinations

Protein determinations were performed by the dye binding method of Bradford (26) using bovine IgG as a standard. The protein concentration was also determined by absorbance using the extinction coefficients for pure recombinant TNF- α and 18 kDa recombinant TNF- β of 1 mg/ml = 1.6 and ϵ_{280} 1 mg/ml = 1.68, respectively.

^{125}I -Labeling of TNFs

Highly purified preparations of natural and recombinant TNF- β were iodinated by the chloramine T oxidation method to a specific activity of 70 μ ci/ μ g (27,28). Recombinant TNF- α was iodinated using the Bolton-Hunter reagent to a specific activity of 25 μ ci/ μ g (29).

Epitope Mapping of Monoclonal Anti-TNF- α Antibodies

Micro ELISA wells (Dynatech removawell) were coated for 16 hours at 4°C with purified anti-TNF- α monoclonal antibodies (200 ng/well) diluted in PBS. The unreacted protein binding sites were blocked by incubating the wells for 30 min with 1 percent gelatin in TBS (150 μ l). ^{125}I -TNF- α (~10,000 cpm) was incubated with a panel of monoclonal antibodies in uncoated microwells for 16 hours at 22°C. Test antibodies that were preincubated with ^{125}I TNF- α were then incubated for 2 hours at 22°C with antibody coated wells. The wells were washed five times with PBS-Tween and the radioactivity bound to each well was measured with a gamma counter.

TNF- α Monoclonal Antibodies Affinity Determination

The affinity of anti-TNF- α monoclonal antibodies was estimated by the method of Muller (30). The radioimmunoassay was performed as follows: The antibodies were diluted in 0.6 ml of TBS, 2 mg/ml gelatin. 0.2 ml ^{125}I -TNF (~10,000 cpm), diluted in TBS, 2 mg/ml gelatin containing 1 percent normal mouse serum, was added and allowed to incubate for 5 hours at 22°C. Goat anti-mouse IgG, diluted in PBS containing 5 percent PEG 8000 (w/v) was added and incubated for 1 hour at 22°C. The tubes were centrifuged at 2500 x g for 30 min, decanted, and the pellets were counted in a gamma counter.

Synthetic Peptides

Peptides were synthesized using a solid phase method as previously described (31).

Production of Monoclonal Antibodies

Initial attempts to immunize mice with small amounts of purified natural TNF- α in Freund's adjuvant alone yielded low titers of antibody in both ELISA and neutralization assays. However, boosting these animals with TNF- α adsorbed onto alum resulted in a large increase in both the serum ELISA and neutralization titers. A combination of alum and Freund's adjuvant boosts produced an even higher serum titer of neutralizing antibodies. For four days prior to fusion, a hyperimmune mouse was boosted daily with recombinant TNF- α . This immunization protocol has been shown to yield large numbers of hybridomas secreting high affinity antibodies (18,19). Splenocytes from this animal were fused with myeloma cells as described in "Materials and Methods". The resulting hybridomas were screened for antibody production with recombinant TNF- α by ELISA, and by inhibition of cytolytic activity. Approximately fifty positive cultures were identified and after cloning by limiting dilution, twelve anti-TNF- α clones were isolated and characterized (Table 1).

TABLE 1
Characteristics of Anti-TNF- α Monoclonal Antibodies*

| Epitope Binding Group | Clone | Neutralizing | Antigen Release Conditions | Isotype | Affinity L/mole |
|-----------------------|-----------------|--------------|-------------------------------|--------------|---------------------|
| I | 5E3-H3 (TNFB) | + | pH 4, 3.5 M KSCN | K γ 1 | 10 ⁹ |
| II | 1A5-E5 (TNFF) | + | None | K γ 1 | 10 ¹⁰ |
| II | 1B2-G3 (TNFD) | + | None | K γ 1 | 10 ¹⁰ |
| II | 5A10-F2 | + | None | K γ 1 | 5 x 10 ⁹ |
| II | 2H10-F12 (TNFE) | + | None | K γ 1 | 10 ¹⁰ |
| III | 3C4-F7 | - | pH 4, 1.25 M KSCN, 3.5 M KSCN | K μ | N.D. ^b |
| IV | 2G3-G6 | + | 1.25 M KSCN, 3.5 M KSCN | K γ 1 | N.D. |
| IV | 5G2-G5 | + | 1.25 M KSCN, 3.5 M KSCN | K γ 1 | N.D. |
| V | 3F10-H4 (TNFG) | - | pH 4, 1.25 M KSCN, 3.5 M KSCN | K γ 1 | N.D. |
| N.T. ^a | 1E2-G2 (TNFA) | - | pH 4, 1.25 M KSCN, 3.5 M KSCN | K γ 1 | N.D. |
| N.T. | 1G2-A2 (TNFC) | + | None | K γ 1 | N.D. |
| N.T. | 2B6-G5 | - | pH 4, 1.25 M KSCN, 3.5 M KSCN | K γ 1 | N.D. |

^a N.T., not tested

^b N.D., not determined, preliminary analysis showed the affinity to be less than 10⁹ L/mole

* The procedures used to determine neutralizing ability, antigen release conditions, isotype and affinity are described in "Materials and Methods".

The immunization of several mice with natural TNF- β (25 kDa form) in Freund's adjuvant resulted in the production of low titer antibodies that reacted in ELISA assays, but failed to neutralize TNF- β biological activity. Repeated boosting failed to elicit neutralizing antibodies. In an attempt to increase immunogenicity, TNF- β was polymerized by treatment with glutaraldehyde. Mice immunized with polymerized TNF- β in Freund's adjuvant produced antiserum against TNF- β with high titers detected by ELISA. However, these animals failed to produce neutralizing antibodies until they were further boosted with TNF- β adsorbed on alum. Splenocytes were isolated from a hyperimmune animal and fused with myeloma cells as described in "Materials and Methods". The resulting hybridomas were screened for antibody production with natural TNF- β (25 kDa species) by ELISA and for neutralization of biological activity. Approximately seventy positive cultures were identified, and after cloning by limiting dilution, thirteen ELISA positive clones were isolated and characterized (Table 2).

TABLE 2
Characteristics of Anti-TNF- β Monoclonal Antibodies*

| Epitope Binding Group | Clone | Neutralizing | Antigen Release Conditions | Isotype | Binding to recombinant 16 kDa TNF- β |
|-----------------------|---------------|--------------|-------------------------------|-------------------------|--|
| I | 2B8-G7 (LTA) | + | pH 4, 1.25 M KSCN, 3.5 M KSCN | K γ ₁ | + |
| I | 1G11-E4 (LTB) | + | pH 4, 3.5 M KSCN | K γ ₁ | + |
| II | 1C4-G6 (LTC) | - | 3.5 M KSCN | K γ ₁ | - |
| II | 1F12-G4 | - | 3.5 M KSCN | K γ ₁ | - |
| II | 2E10-H1 | - | 3.5 M KSCN | K γ ₁ | - |
| II | 2F12-F4 | - | 3.5 M KSCN | K γ ₁ | - |
| II | 2G2-F7 | - | 3.5 M KSCN | K γ ₁ | - |
| II | 3B5-H5 | - | 3.5 M KSCN | K γ ₁ | - |
| II | 2A9-EB | - | 3.5 M KSCN | K γ ₁ | - |
| III | 1D9-E11 (LTD) | - | 3.5 M KSCN | K γ ₃ | + |
| III | 2D9-B1 | - | 3.5 M KSCN | K μ | + |
| III | 4E12-H9 | - | 3.5 M KSCN | K γ ₁ | + |
| III | 4H8-G8 | - | 3.5 M KSCN | K γ ₁ | + |

* The procedures used to determine neutralizing ability, antigen release conditions, isotype and binding to 16 kDa TNF- β are described in "Materials and Methods".

Various monoclonal antibodies were tested for binding to TNF- α and TNF- β by ELISA, and for neutralization of cytolytic activity. Both natural and recombinant TNF- α exhibit an apparent molecular weight of 17 kDa on SDS-PAGE gels (11). In ELISA assays, all of the anti-TNF- α monoclonal antibodies bound equally well to natural and recombinant TNF- α , but did not bind to recombinant murine TNF (32). The failure of monoclonal antibodies produced against human TNF- α to react with TNF from other species has been observed by others (33). Four different clones produced high affinity antibodies which ranged in affinity from 10^{10} to 5×10^9 L/mole (Table 1). Most of the high affinity antibodies could bind to TNF- α at high and low pH and in the presence of 3.5 M KSCN (Table 1). Eight of the twelve monoclonal antibodies could completely neutralize TNF- α activity (Table 1). The best neutralizing monoclonal antibody, clone 1D9-E11, had a neutralization titer of 2700 units TNF- α neutralized/ μ g of purified IgG. The neutralizing antibodies required an incubation of antigen and antibody for 1 hour at 37°C or 16 hours at 4°C to completely neutralize TNF- α cytolytic activity. None of the TNF- α specific monoclonal or polyclonal antibodies either neutralized or bound to TNF- β , even when tested at concentrations greater than one hundred times the amount required to show neutralization or binding to TNF- α .

In contrast to TNF- α , natural TNF- β , from mitogen stimulated lymphocytes or RPMI 1788 cells, is glycosylated and exists as 25 kDa and 20 kDa forms (9-10). The 20 kDa form is a truncated version of the 25 kDa TNF- β and lacks twenty-three amino acids from the amino terminus (10). When produced in *E. coli*, the 25 kDa and 20 kDa forms of TNF- β lack glycosylation and exhibit molecular weights of 18 kDa and 16 kDa, respectively on SDS-PAGE (12). Natural and recombinant forms of full length and truncated TNF- β are biologically active in cell lysis bioassays (10,12). All of the anti-TNF- β monoclonal antibodies bound to natural 25 kDa and recombinant 18 kDa TNF- β equally well in ELISA assays, indicating that none of the antibodies produced were specific for a carbohydrate moiety. Seven of the thirteen monoclonal antibodies did not bind 16 kDa TNF- β , and were specific for the 18 kDa form, suggesting that these antibodies are specific for the amino terminus of TNF- β (Table 2). Two clones were isolated that produced neutralizing antibodies (Table 2). These antibodies could neutralize the activity of the recombinant 18 kDa as well as recombinant 16 kDa TNF- β . Monoclonal antibody, clone 1G11-E4, had a neutralization titer of 550 units TNF- α neutralized/ μ g of purified IgG. Clone 2B8-G7 was substantially less, and could only neutralize about 40 percent of the TNF- β activity even when used at high concentrations. The neutralization of TNF- β cytotoxic activity was time and temperature dependent, and was maximal after an incubation for 16 hours at 37°C. Only a partial neutralization of biological activity was observed after incubation with TNF- β for 16 hours at 4°C or for 1 hour at 37°C even with an excess of antibody. Anti-TNF- β monoclonal and polyclonal antibodies neither bound nor neutralized TNF- α , even when tested at concentrations greater than one hundred times the amount necessary to show binding or neutralization of TNF- β .

Epitope Mapping of TNF- α and TNF- β Using Monoclonal Antibodies

The number of distinct TNF- α antigenic epitopes, defined by the panel of monoclonal antibodies, was determined by ELISA using an antibody competition method as described in "Materials and Methods". Five epitopes were identified, three of which reacted with neutralizing antibodies. Anti-TNF- α monoclonal antibodies were categorized into five groups based on their binding to these epitopes as shown in Table 1. Members of both groups I and II are neutralizing antibodies, and can bind simultaneously to TNF- α . Group III consists of one non-neutralizing antibody that partially inhibits the binding of neutralizing group I antibodies. Group IV consists of neutralizing antibodies that partially inhibit neutralizing group II antibodies. Finally, group V consists of one antibody that is non-neutralizing, does not affect group I or II binding and can react with denatured TNF- α on western blots. It

is interesting to note that all of the members of an epitope binding group have the same antigen release conditions (Table 1). These conditions are probably determined by the nature of the antigen-antibody interaction and may be similar for all antibodies binding to a given epitope.

Radiolabeled TNF- β did not bind well to several anti-TNF- β monoclonal antibodies in radioimmunoassays or solid phase antibody binding experiments, although greater than 90 percent of the labeled TNF- β could be precipitated by polyclonal antibodies. The lack of binding, probably a result of damage or alteration of the antigen binding sites by the iodination reaction, or because the anti-TNF- β antibodies exhibited low affinity binding, precluded the same epitope binding analysis performed using anti-TNF- α monoclonal antibodies. It can be inferred, however, that the panel of anti-TNF- β monoclonal antibodies bind to at least 3 antigenic epitopes (Table 2). Group I consists of two neutralizing antibodies, group II, antibodies that react with 18 kDa TNF- β but not 16 kDa TNF- β , and group III, antibodies that react with both forms of TNF- β but fail to neutralize biological activity.

Cross-Reactivity of Monoclonal Antibodies with Synthetic Peptides

In an attempt to define the active sites of TNF- α and TNF- β and to precisely define their antigenic domains, we examined several synthetic peptides for their reactivity with anti-TNF monoclonal antibodies. The peptides correspond to some of the regions of homology between TNF- α and TNF- β , and one peptide corresponds to the amino terminal region of TNF- β which has been reported to be non-essential for cytolytic activity (11). The relationship of these peptides to TNF- α and TNF- β is shown in Figure 1. None of the monoclonal antibodies cross-reacted with any of the TNF- α peptides, residues 1-14, 1-30, 15-30 and 39-66 or with the TNF- β peptides, residues 35-57, 82-94 and 139-155. A group of seven different antibodies cross-reacted with a TNF- β peptide, residues 7-19. All of these antibodies were non-neutralizing and were specific for recombinant 18 kDa TNF- β and did not bind to the recombinant 16 kDa form (Table 2), providing further evidence that the amino terminus of TNF- β is not involved in biological activity.

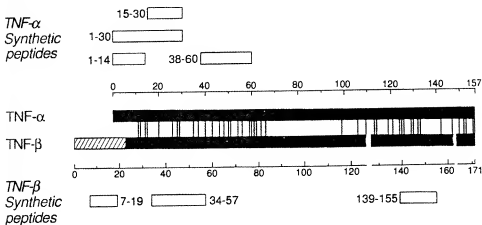


FIGURE 1. Schematic Diagram of TNF- α and TNF- β Proteins Showing Homologous Regions and the Location of Synthetic Peptides. The TNF- α and TNF- β proteins are represented by the horizontal black bars and are numbered by amino acid starting from the amino terminal ends. The hatched region of TNF- β represents the N-terminal 23 amino acids that are missing in the 20 kDa natural and 16 kDa recombinant molecules. The vertical lines between two bars represent homologous amino acids. Synthetic peptides are represented by open boxes and their position corresponds to the sequence as indicated.

Various monoclonal antibodies were examined for their binding to SDS-denatured TNFs using the immunoblot technique (25). A single TNF- α specific antibody and seven anti-TNF- β antibodies were identified that could bind to denatured recombinant TNF- α or TNF- β , respectively (Fig. 2). The anti-TNF- α antibody (3F10-H4) did not cross-react with any of the TNF- α synthetic peptides, was non-neutralizing and bound to a unique epitope of TNF- α that was not cross-reactive with any of the other TNF- α specific antibodies (Table 1). The anti-TNF- β antibodies all shared the same specificity. These antibodies were specific for the amino-terminus of recombinant 18 kDa TNF- β (Table 2), cross-reacted with the TNF- β synthetic peptide, residues 7-19 and were non-neutralizing. These results indicate that many of the anti-TNF monoclonal antibodies, including all of the neutralizing antibodies, probably bind to discontinuous epitopes which are disrupted by treatment with SDS. Because these neutralizing antibodies recognize a precise conformation, it is unlikely that they can react with inactive TNFs.

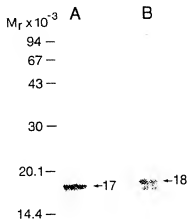


FIGURE 2. TNF Immunoblots Probed with the Monoclonal Antibodies. Recombinant TNF- α reacted with antibody 3F10-H4 (A). Recombinant 18 kDa TNF- β reacted with antibody 1C4-G6 (B). (All of the monoclonal antibodies that were specific for 18 kDa TNF- β gave results identical to 1C4-G6). The positions of the TNF- α and TNF- β bands are shown by the arrows designated 17 and 18, respectively, which indicate the estimated molecular weight in daltons $\times 10^{-3}$.

Immunoaffinity Purification of TNF- α and TNF- β

Two monoclonal antibodies, clone 5E3-H3 and clone 1G11-E4, were chosen for the immunoaffinity purification of TNF- α and TNF- β , respectively. Both antibodies were neutralizing, and their binding to antigen could be reversed using conditions that did not destroy the biological activities of TNF- α or TNF- β . As shown in Figure 3, 5E3-H3 antibodies exhibit reduced affinity for TNF- α below pH 4.5, whereas 1G11-E4 antibodies show reduced binding below pH 6.0 or above pH 9.5. Immunoabsorbents were prepared by coupling purified monoclonal antibodies to cyanogen bromide activated Sepharose as described in "Materials and Methods". About 90 percent of the antibodies were bound to the

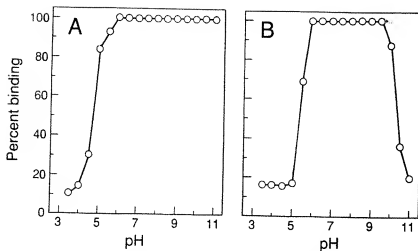


FIGURE 3. The Effect of pH on the Binding of 5E3-H3 Antibodies to TNF- α (A) and 1G11-E4 Antibodies to TNF- β (B). The binding was measured by ELISA as described in "Materials and Methods". Percent binding is relative to the binding at pH 7.4 which represents 100 percent.

gel and the immobilized antibodies retained 50 percent of their theoretical antigen binding capacity.

The anti-TNF- α monoclonal antibody column provided a substantial purification of recombinant TNF- α in a single chromatographic step. A typical purification profile is shown in Figure 4(A). Prior to immunoaffinity chromatography, TNF- α was partially purified from *E. coli* cell lysates by

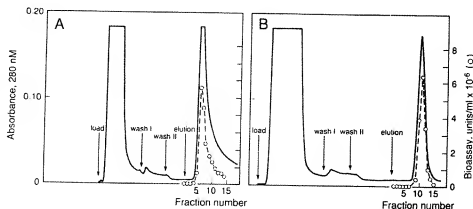


FIGURE 4. Immunoaffinity Column Chromatographic Profiles for TNF- α (A) and TNF- β (B). A column of anti-TNF- α (antibody 5E3-H3) Sepharose (4.5 x 1.6 cm) was loaded at a flow rate of 9.5 cm/hr with 0.5 ml of a 50 percent saturated ammonium sulfate fraction of an *E. coli* lysate containing recombinant TNF- α . The column was washed at a flow rate of 19 cm/hr with TBS containing 0.05 percent Tween 20 (wash I) and then TBS alone (wash II). Bound TNF- α was eluted at a flow rate of 9.5 cm/hr with 0.15 M NaCl, 50 mM Na acetate, pH 4.5, and 2 ml fractions were collected into 0.2 ml NaCl, pH 8.5. A column of anti-TNF- β (antibody 1G11-E4) Sepharose (9.3 x 1.6 cm) was loaded with 5 ml of a 40 percent saturated ammonium sulfate fraction of an *E. coli* lysate containing recombinant TNF- β . The washes and elution conditions are the same as those used for TNF- α . The activity of the purified TNF- α and TNF- β fractions was measured using a standard L-929 cell lysis assay.

precipitation with ammonium sulfate and the reconstituted precipitate was filtered. This pretreatment prevented the accumulation of a colored material on the immunoaffinity column and prevented clogging. Dialysis of the dissolved ammonium sulfate precipitate was not necessary. When this crude bacterial extract fraction was loaded on the antibody column, most of the *E. coli* proteins passed through, and all of the TNF- α activity was retained. The addition of 0.05 percent Tween 20 in the column washes was necessary to remove minor contaminants that adsorbed non-specifically to the column matrix. The bound TNF- α was eluted from the column using a pH 4.5 buffer. We found that TNF- α and TNF- β are not stable at pH 4.5 for more than a few hours at 22°C, therefore, the eluted fractions were collected into 1/10 volume of 1M Tris pH 8.5 to adjust the pH of the eluted TNF- α to 7.8 in order to insure the stability of biological activity. Immunoaffinity purified TNF- α was greater than 98 percent pure by SDS-PAGE (Fig. 5) and had a specific activity of 2.9×10^7 cytolytic units/mg (Table 3), which is in good agreement with the value of 3.0×10^7 units/mg obtained for TNF- α purified from natural resources by conventional chromatography (11). In a single chromatographic step, TNF- α could be purified 21-fold from *E. coli* lysates (Table 3).

The anti-TNF- β monoclonal antibody column could be used to purify both forms of recombinant TNF- β . The purification protocol used for TNF- β was essentially the same as that used for TNF- α . A typical chromatographic profile of a TNF- β purification (18 kDa form) is shown in Figure 4(B). Immunoaffinity purified 18 kDa TNF- β was greater than 99 percent pure by SDS-PAGE and had a specific activity of 3.9×10^7 cytolytic units/mg (Table 3), compared with 4.0×10^7 units/mg reported for TNF- β purified from natural sources by conventional chromatography (9). In comparison to a typical TNF- α immunoaffinity purification of 21-fold, TNF- β was purified 285-fold from *E. coli* lysates (Table 3). This difference is due to the relatively higher purity of the TNF- α starting material (Fig. 5). An anti-TNF- β column was used over twenty-five times without any detectable loss in capacity or any change in the purity in the eluted TNF- β . This result indicates that no leaching of immobilized antibody was detected, and that the mild elution conditions used did not damage the antibody binding activity.

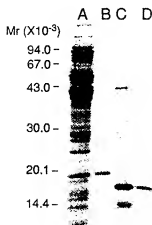


FIGURE 5. Analysis of Immunoaffinity Purified TNFs by SDS-PAGE. 100 μ g of TNF- α immunoaffinity column load (lane A), 2 μ g of immunoaffinity purified TNF β (lane B), 25 μ g of TNF- α immunoaffinity column load (lane C), 2 μ g of immunoaffinity purified TNF- α (lane D). The samples were run on a 12 percent polyacrylamide gel under reducing conditions and stained with Coomassie Blue R-250. By laser densitometry, the purity of immunoaffinity purified TNF- α and TNF- β was determined to be greater than 98 percent and greater than 99 percent, respectively.

TABLE 3
Immunoaffinity Purification of Recombinant TNF- α and TNF- β

| Procedure | Protein ^a | Activity ^b | Specific Activity | Yield | Purification |
|---|----------------------|--|--------------------------------|---------|--------------|
| | mg | units ₇ x 10 ⁻⁷ | units/mg x 10 ⁻⁶ | Percent | -fold |
| TNF- α | | | | | |
| E. coli lysate | 52 | 7.1 | 1.4 | 100 | 1 |
| 50 percent saturated ammonium sulfate precipitation | 18 | 7.1 | 3.9 | 100 | 2.9 |
| Antibody (TNF β) Sepharose | 1.4 | 4.1 | 29.0 | 57.7 | 21.4 |
| TNF- β | | | | | |
| E. coli lysate | 220 | 3.0 | 0.14 | 100 | 1 |
| 40 percent saturated ammonium sulfate precipitation | 66.5 | 3.0 | 0.45 | 100 | 3.3 |
| Antibody (LT β) Sepharose | 0.9 | 3.5 | 39.0 | 117 | 285 |

^a The protein concentration was determined by the method of Bradford (26).

^b Activity was determined using an L-929 cell cytotoxicity assay (11).

Assay of TNF- α and TNF- β Using Specific ELISA Assays

Specific double sandwich ELISA assays were developed for both TNF- α and TNF- β . Purified neutralizing monoclonal antibodies, anti-TNF- α clone 1B2-63 or anti-TNF- β clone 1G11-E4, were adsorbed to plastic microELISA wells and utilized to specifically bind TNF in the sample solution. A combination of rabbit polyclonal anti-TNF- α or anti-TNF- β antibodies and HRP conjugated anti-rabbit IgG was then used to detect bound antigen. After incubation with a o-phenylenediamine substrate solution, the absorbance of each well was measured with an automated plate reader and a desktop computer was used for data reduction. Typical standard curves for a TNF- α and a TNF- β ELISA are shown in Figure 6. Both assays had useful ranges of about 0.4 to 25 ng/ml TNF. The specificity of each assay was demonstrated by the lack of any signal generated by 30 μ g/ml of TNF- β in a TNF- α ELISA or by 30 μ g/ml TNF- α in a TNF- β ELISA.

To compare the results obtained from TNF- α and TNF- β ELISA assays with the bioassay, we measured TNF levels in column fractions from typical immunoaffinity purifications of TNF- α and TNF- β using both types of assays (Fig. 7). The ratio of bioactive to immunoreactive TNFs i.e., the specific activity, of all of the TNF- α and TNF- β fractions was relatively constant. The values for the specific activities of TNF- α and TNF- β obtained by this method were $1.7 \pm 0.34 \times 10^7$ units/mg and $3.0 \pm 1.0 \times 10^7$ units/mg, respectively. This result demonstrates that there was no significant difference in the levels of TNF- α and TNF- β measured by ELISA or by bioassay, and that the ELISA assays probably only detect bioactive TNFs.

DISCUSSION

In this report, we described the production, characterization, and application of specific monoclonal antibodies against two very similar

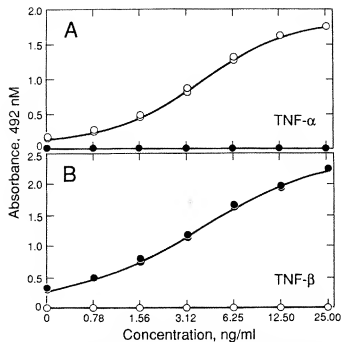


FIGURE 6. Typical Standard Curves for a TNF- α ELISA (A) and a TNF- β ELISA (B). Open circles represent TNF- α and closed circles represent TNF- β . Up to 30 μ g/ml of either TNF- α or TNF- β failed to react in a reciprocal assay.

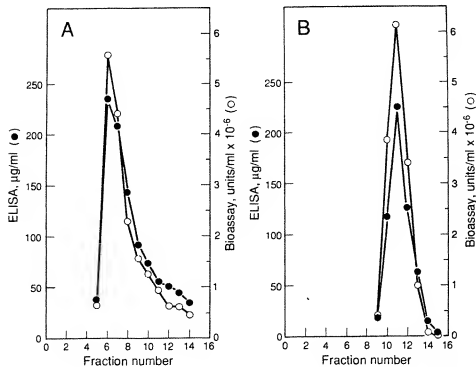


FIGURE 7. Comparison of ELISA Assays with a Standard L-929 Cell Lysis Assay. Fractions from a typical TNF- α (A) or TNF- β (B) immunoaffinity chromatography purification assayed by ELISA and by bioassay.

cytokines, TNF- α and TNF- β . Monoclonal antibodies, as well as rabbit polyclonal antibodies, were highly specific and showed no cross reactivity between these cytolytic proteins. This indicates that despite the fact that these cytokines share substantial amino acid identity, and bind to the same cell surface receptor, their antigenic epitope structures are quite different. We isolated three types of neutralizing anti-TNF- α monoclonal antibodies that bound to distinct antigenic epitopes, including two classes of neutralizing antibodies that could bind simultaneously. These results indicate that TNF- α biological activity is probably not neutralized by antibodies binding to a unique active site, but probably by interfering with the binding of TNF to its cell surface receptor. Non-neutralizing monoclonal antibodies also failed to cross react with both TNF- α and TNF- β , demonstrating that despite their high degree of homology, no antigenic epitopes are shared between these cytokines or that antibodies to shared epitopes are simply rare. We attempted to define the active sites and the antigenic epitopes of TNF- α and TNF- β using synthetic peptides. Most of the antibodies that were isolated were conformation specific, and it was not possible to identify TNF antigenic epitopes by this approach. However, several anti-TNF- β monoclonal antibodies were identified that reacted with amino-terminal sequences of TNF- β that were not required for biological activity. This region may represent the most immunogenic epitope of TNF- β and explain our difficulty in raising neutralizing antibodies in mice.

Various monoclonal antibodies were used to purify milligram quantities of TNF- α and TNF- β , eliminating the need for complicated multi-column procedures. These antibodies were selected because they were neutralizing, and, therefore, likely to bind only to active TNFs, and had reduced affinity for TNFs under acidic conditions that did not destroy cytolytic activity. By using these mild elution conditions, the immunoaffinity columns could be reused many times with no change in performance. A TNF- β specific column was used to purify both recombinant 18 kDa 16 kDa TNF- β . It is likely that this method can be used to purify other engineered variants of recombinant TNFs, eliminating the need for a new purification process for each variant.

TNF monoclonal and polyclonal antibodies were used to develop sensitive sandwich ELISA assays. The TNF ELISA assays were as sensitive as the bioassay, but the ELISA assays could discriminate between TNF- α and TNF- β , which the bioassay cannot, and were faster and easier to perform than the bioassay.

The monoclonal antibodies to both TNF- α and TNF- β described here should prove to be useful in the study of the mechanisms of action of these cytokines. Sensitive ELISA assays can be used to identify and quantitate TNFs and neutralizing antibodies can selectively neutralize TNF cytolytic activity in *in vitro* systems, isolating the effects of TNFs from interferons, lymphokines, and other effector molecules. Recently, several biological activities besides tumor cell killing have been ascribed to tumor necrosis factors. For example, TNF- α has been shown to be anti-parasitic (34) and anti-viral (35,36), and it may play a role in cachexia (37), endotoxin shock (38) and inflammation (39). Both TNF- α and TNF- β can cause bone resorption *in vitro* (40). The observation that myeloma cell lines can produce TNF- β has led to the speculation that TNF- β may be involved in the bone destruction often seen in myeloma patients (40). It is not known whether all of these activities are mediated by TNF binding to the same cell surface receptor. Although, it has been shown that neutralizing TNF- α specific monoclonal antibodies can also inhibit the action of TNF- α on lipoprotein lipase activity in adipocytes. This enzyme is thought to play a key role in cachexia. The availability of monoclonal antibodies against several epitopes of both TNF- α and TNF- β may help to clarify this issue. In addition, the role of TNFs under normal physiological conditions, in tumor killing and under pathological conditions is not yet understood. Specific ELISA assays that can identify and quantitate TNFs will aid in elucidating the role of TNFs in normal and disease states. Finally, neutralizing anti-TNF monoclonal antibodies may be able to block the side effects of TNF *in vivo* providing a specific therapy for TNF mediated disorders.

ABBREVIATIONS: BSA, bovine serum albumin; CFA, complete Freund's adjuvant; DMEM, Dulbecco's modified Eagle's minimal essential medium; EDTA, ethylenediaminetetraacetate; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HRP, horseradish peroxidase; ID, intradermally; IM, intramuscularly; IP, interperitoneally; IV, intravenously; IFA, incomplete Freund's adjuvant; LT, lymphotoxin; NZW, New Zealand white; PEG, polyethyleneglycol; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SC, subcutaneously; TBS, tris buffered saline; TNF, tumor necrosis factor.

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Complement

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The term "complement" is used to include a complex group of interacting blood proteins and glycoproteins found in all vertebrates. These proteins have as their primary functions the production and regulation of inflammation, the opsonization of foreign materials for phagocytosis, and the mediation of direct cytotoxicity against various cells and microorganisms. The first evidence for the existence of such a system arose in the late 19th century during studies of the mechanism of host defense against invading bacteria and studies of the mechanism of destruction of foreign or mismatched transfused cells (1-3). These studies demonstrated that individuals are ca-

pable of responding to invading microorganisms or to injected foreign cells by the production of antibody. Such antibodies are able *in vitro* to agglutinate the organisms or foreign cells used in the immunization but are unable to mediate cell death. It was discovered that the addition of fresh serum to a mixture containing specific antibody and the microorganism or immunizing cell often led to cell death. The importance of this property of fresh serum was quickly recognized, and a series of investigations was begun to define the biochemical and biologic basis of the phenomenon of cell lysis.

Work over the next several decades demonstrated the

complex nature of the lysis phenomenon. It quickly became clear that the lytic material, present in all fresh sera, was not a simple substance but could be separated into several principles by even the crude chemical techniques then available. For example, dialysis of the serum against water separated the lytic material into a precipitable euglobulin fraction and a soluble pseudoglobulin fraction. Neither fraction had lytic activity when tested, but mixing the fractions restored this activity. The materials that acted together to produce this cytotoxic response were collectively termed alexin by Bordet, and first "addiment," and later complement by Ehrlich. Although some early workers doubted that the reactions leading to lysis followed simple chemical rules, it was soon recognized that this was indeed the case and experimental systems were established to permit detailed study of these biochemical events. Many of the experimental models established at the turn of the century are still in use today and it is valuable to consider a few of them in some detail.

Much of the early work was directed toward establishing an *in vitro* system designed to allow for analysis of each of the steps involved in complement-mediated cell death. Erythrocytes from many species were screened to determine which were the most easily lysed by antibody and complement. Sheep erythrocytes proved to be particularly useful since, when sensitized with antibody, they were highly susceptible to the lytic action of complement. It was also discovered that sheep erythrocytes have on their surface a potent lipopolysaccharide antigen (termed Forssman antigen) (4) and that high titered antibody to this antigen could be prepared conveniently in the rabbit. In general, sera from all mammals could be used as a complement source, but the degree of lysis varied greatly among different species. It was found that fresh guinea pig serum was the most potent lytic serum easily available. A test system utilizing sheep erythrocytes sensitized with rabbit antibody for studies of lysis in fresh guinea pig serum was commonly employed. With time, it became possible to create specific intermediates bearing various complement components on the surface of the antibody-coated sheep erythrocytes and to study the interaction of each newly defined complement protein with the appropriate cellular intermediate in the complement sequence. Early efforts focused almost exclusively on the events that occur in the lysis of the antibody-coated sheep red cell. Such events now comprise the classical complement pathway. In more recent years it has become clear that another closely related series of proteins is often involved in the lysis of bacteria with or without the intervention of antibody. These bacteriolytic proteins make up the alternative complement pathway. The two pathways of activation converge at the step of C3 activation and engage the later lytic components in the complement cascade (Fig. 1). The classical pathway, in general, is initiated by the formation of an antigen-antibody complex. Recognition of the antigen-antibody complex by the proteins of the classical pathway leads to sequential formation of enzymes with serine protease activity. These cleave and activate C3. The proteins of the alternative pathway mediate this same end result, albeit with slower kinetics of activation. Cleaved C3 (C3b) interacts with the C3-cleav-

ing enzymes of either the classical or alternative pathways and alters their substrate specificities such that they are able to cleave C5. Cleaved C5 (C5b) then interacts with the remaining numbered components, C6, C7, C8, and C9, and these five terminal complement components, acting in concert, mediate cell lysis. This general scheme of complement activation is presented in Fig. 1.

The complement system is essentially entirely conserved throughout the mammalian species that have been studied, and the proteins are often (but not always) interchangeable across species. The system is by far best characterized in the human and guinea pig, although much data on the murine proteins have evolved recently through molecular techniques.

TERMINOLOGY

The nine proteins of the classical pathway are designated by an uppercase letter C followed by a number. The numbers generally follow the order of action of the components, with the exception of C4, which acts before C2 and C3. Components acting solely in the alternative pathway are designated by letters. Regulatory proteins are designated by a descriptive title (e.g., C4 binding protein) or, in the case of those proteins closely associated with the alternative pathway, a letter (e.g., factor H). Single components or multicomponent complexes that have enzymatic activity are designated by a bar over the component(s) in question (e.g., $\overline{\text{C1r}}$). Molecules that have lost activity through chemical denaturation or by the action of a control protein are usually designated by a prefix lower case i (e.g., iC3). Fragments or subunits of the various components are designated by a lowercase letter suffix (e.g., C3b).

THE CLASSICAL COMPLEMENT PATHWAY

The Role of Immunoglobulin

Activation of the classical pathway is initiated by the binding of C1, the first component in the cascade, to an antigen-antibody complex and the subsequent activation of the antibody-bound C1 (5,6). The steps have been examined in considerable detail. Not all classes of antibody are capable of binding C1 to initiate the classical pathway. IgG and IgM antibodies have this ability, but IgE, IgD, and IgA antibodies do not (5). Studies in a number of test systems have demonstrated that a single molecule of IgM bound to a particulate antigen is capable of binding one molecule of C1, a complex zymogen protease (7). However, the processes of antibody binding of C1 and the activation of C1 to a protease capable of cleaving C4 and C2 are not equivalent (6). To mediate C1 activation, the IgM antibody molecule must engage the antigen by more than one of its Fab arms. This was inferred from studies of the binding and activation of C1 on anti-hapten IgM sensitized sheep erythrocytes coated with various den-

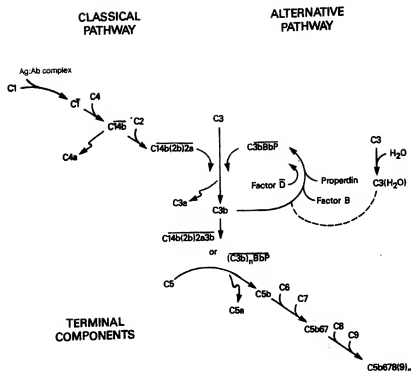


FIG. 1. A schematic diagram of complement activation. Regulatory proteins, side reactions, and inactive fragments have been omitted for clarity. Enzymatically active species are designated by *overbars*.

sities of a defined hapten, and by analysis of C1 activation by idiotype anti-idiotype immune complexes containing IgM (8,9). The finding that multiple variable region sites must be bound to an antigenic surface for C1 activation suggests that binding facilitates a conformational change in the antibody that promotes C1 activation.

On the other hand, in most of the model systems studied, two IgG molecules side by side (a doublet) are required for C1 binding and activation (10). In molecular terms, the requirement for an IgG doublet greatly reduces the efficiency of IgG as compared to IgM in inducing classical pathway activation. In most systems that examine the lysis of target particles, hundreds or thousands of IgG molecules must be supplied before, by chance, two molecules come to lie sufficiently close together on a surface to produce a doublet. If the distribution of antigen molecules on a surface precludes the possibility of two IgG molecules coming to lie sufficiently close together, the IgG may not activate complement at all. In fact, it has been shown that IgA, under some circumstances, blocks complement activation by IgG antibody by inhibiting the formation of doublets (11). Not all IgG subclasses are capable of activating the classical pathway. Human IgG1, IgG2, and IgG3 are all activators of the classical pathway, but IgG4 is not. In mouse systems, $\gamma 2a$, $\gamma 2b$, and $\gamma 3$ activate the classical complement pathway, and in guinea pig, $\gamma 2$ antibodies activate the classical pathway.

It is not known whether the binding of IgG antibody to its substrate causes a structural change in the antibody that induces increased affinity for C1. Binding of C1 to surface-bound IgG doublets may simply be a consequence of cooperative binding effects. It has been shown that IgG monomer will interact with native C1, albeit weakly (12).

A cluster of closely associated IgG molecules may allow for multiple points of contact between C1 and the activator. In the case of IgG it has been suggested that not only must the CH₂ domain bind C1 but there must be a second point of contact with the CH₁ domain for activation to occur (15). In keeping with this, it is also suggested that a rather narrow range of angles between the Fab arms of IgG are optimal for C1 activation (8). Accordingly, both simple spatial clustering and structural rearrangements induced by antigen binding may have a role in C1 activation by IgG. Recently, a C1-binding motif consisting of a trio of charged amino acid residues has been identified in the CH₂ domain of murine IgG. These residues are highly conserved in most mammalian IgGs examined and appear to be necessary, but not sufficient, for efficient C1 binding (16).

C1

C1 exists in serum as a three-subunit macromolecule with the subunits held together in the presence of ionic calcium (7,17). C1q is the subunit that binds to an antigen-antibody complex via the antibody CH₂ domain; it has a molecular weight (MW) of about 400,000 and is composed of 18 chains: three chain types termed A, B, and C with six copies of each per molecule. The protein has a central

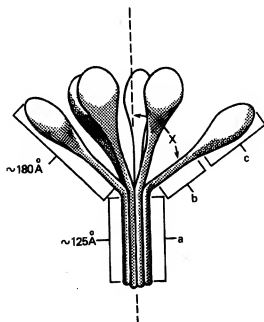


FIG. 2. A conceptualization of C1q. The central core is designated by (a) and the collagen-like fibrillar arms by (b). The globular heads which bind to immunoglobulins are designated by (c). Angle X ranges from 20° to 80°.

core and six radiating arms, each of which ends in a pod-like globular structure (Fig. 2). The amino terminal end of each arm has a triple-helix, collagen-like structure and can be cleaved by bacterial collagenases. These regions are rich in glycine, hydroxylysine, and hydroxyproline. Binding of C1q to the CH₂ domain of the antibody occurs through the podlike carboxyl terminus at the end of each arm. Potentially, each of the six C1q arms can bind to one CH₂ domain; it is assumed that multiple C1q-antibody interactions are required for firm binding (see previous discussion). C1q is found in serum in association with two additional C1 subunits: C1r and C1s. C1r and C1s both have molecular weights of about 85,000 and are single-chain proenzymatic forms of serine proteases. Current models envision the two C1r molecules and the two C1s molecules arranged linearly with the two C1r molecules in contact in the center of the claim and the two C1s molecules at the ends (18). When C1 is incubated in ethylene diamine tetraacetate (EDTA)-containing buffers to remove calcium, the molecule dissociates. The two chains of C1r molecules remain associated and the two C1s chains are free in solution. C1q remains intact but loses its C1r and C1s.

The C1r₂-C1s₂ tetramer is believed to associate with the collagenous regions of the C1q molecule when calcium is present (18). Following binding of the intact macromolecular zymogen form of C1 to an antigen-antibody complex, the C1 undergoes enzymatic activation to become an active serine protease. Activation is associated with cleavage of each of the C1r chains and each of the C1s chains into a heavy chain of 57,000 and light chain

of 28,000 daltons. The enzymatic sites reside in each of the smaller subunits in both cases (19).

In *in vitro* studies, activated C1r is capable of cleaving and activating C1s; activated C1s has broader enzymatic specificity and is the enzyme responsible for the cleavage of both C4 and C2. The mechanism by which the binding of C1q leads to activation of C1r and C1s is unknown. Some have suggested that this involves an intramolecular rearrangement within the intact C1 molecule. The mechanism of C1r cleavage of C1s is also unknown. Although some investigators have suggested that activated C1r cleaves the C1s chains within a single macromolecular C1 molecule, others have hypothesized that C1r cleavage of C1s is actually an intermolecular event requiring the proper alignment of two C1 molecules on an activating surface.

C4

The binding and activation of C1 leads to the generation of an enzyme capable of coordinating with and cleaving the second protein in the cascade, C4. C4 is composed of three disulfide-linked chains termed α , β , and γ with MWs of 93,000, 78,000, and 33,000, respectively. The protein is synthesized as a single-chain precursor (proC4) and the three-chain structure is formed as a post-translational event (20,21).

On interaction with C1s, the C4 α chain is cleaved with release of a small fragment, C4a (9,000 daltons), from its amino terminus. C4a is discussed in detail in the section on anaphylatoxins. The larger fragment, C4b, contains the modified α chain (α'), β , and γ and continues the complement cascade. The binding of C4b to a surface, unlike the binding of C1, proceeds via formation of a covalent ester or amide bond and is apparently highly analogous to C3b binding (see later discussion) (22). An antibody site with a bound, active C1 will cleave multiple C4 molecules, and a cluster of C4 molecules will bind to the region surrounding the antibody-C1 site. This represents an amplification step in classical pathway activation since a single C1-fixing site leads to the activation of multiple C4 molecules. Not all deposited C4 molecules are equally active hemolytically (23). Those which bind to, or close to, the antibody-C1 complex will continue the complement cascade. Bound C4b appears to protect adjacent C1 molecules from the action of C1 inhibitor (24). This serves to promote complement activation at the site of an immune complex and limits the effect of nonspecific activation of C1. The binding of C4b to certain targets may have an effect on biologic activity. For example, certain viruses may be neutralized by the deposition of multiple C4 molecules on their surface, preventing their binding to a suitable host cell (25).

C2

The third protein in the antigen-antibody recognition steps of the classical pathway is C2. This molecule con-

sists of a single peptide chain of 95,000 daltons. In the presence of Mg^{2+} ion, C2 binds to C4b and is cleaved by adjacent C1s. Two fragments are formed. A small fragment (C2b, MW 30,000) is cleaved from the molecule, and the larger fragment C2a remains associated with C4b to continue the cascade (26). The complex of C2a and C4b is endowed with new enzymatic activity: the ability to coordinate with and to cleave C3. The active enzymatic site, again a serine protease, resides on the C2a portion of the molecule. Some data suggest that the C2b remains as part of the complex, acting as the C4b-binding site (27). The C4b in the complex binds the C3 molecule and makes it accessible to C2a cleavage. The C4b2a complex, termed the classical pathway C3 convertase, is labile and undergoes decay with physical release of the C2a as an enzymatically inactive fragment. The C4b left behind on the antigenic surface can bind another C2 molecule, which on cleavage by C1 will again form the C3 convertase enzyme.

REGULATION OF THE CLASSICAL PATHWAY

C1 Inhibitor

The C1 inhibitor (C1INH) is a single-chain serum glycoprotein of 105,000 daltons with an unusually high carbohydrate content (about 35 to 40%) (28). The protein functions by combining in 1:1 stoichiometry with the active site on each activated C1r and C1s to destroy its protease activity. Since there are two C1r protease sites and two C1s protease sites per C1 molecule, one molecule of activated C1 can, in theory, react with four molecules of C1-INH. Binding of C1-INH to activated C1 induces disassembly of the C1 molecule with release of two molecules of a C1rC1s (C1INH)₂ complex (29,30). The C1q is presumably left behind on the activating surface where it may interact with additional plasma C1r and C1s or with specific C1q receptors on a variety of cells (see section on receptors). C1-INH chemistry has been examined in considerable detail. It is a member of the family of proteins termed serpins (serine protease inhibitors) and acts by presenting a bait sequence to the enzyme to be inhibited. In the case of the C1-INH, this bait sequence contains a critical arginine at position 444 (31). The enzyme to be inhibited cleaves the C1-INH at the active-site arginine into two fragments of 96,000 and 9,000 MW. Upon cleavage, a reactive site is exposed in the 96,000-dalton fragment which binds to the active site on the enzyme to be inhibited, forming a stable complex that is resistant to boiling in SDS (28). C1-INH is also reported to interact with C1 before its activation. This interaction appears to inhibit C1 activation by spontaneous autocatalysis or non-immune activators but not by antigen-antibody complexes (32). Interestingly, binding of C1-INH to C1r leads to loss of detectable C1r antigen using most anti-C1r antisera. Thus disappearance of antigenically detectable C1r, along with the appearance of cleaved C1r and C1s chains on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), allows one to follow the kinetics of C1 activation and inhibition (33).

C4b Regulation and C4-Binding Protein

The activity of C4b is under the control of a series of membrane-bound and fluid-phase proteins. In this section we describe interactions that can occur in the fluid phase. A separate section discusses all the membrane-bound complement regulators.

C4-binding protein (C4BP) is an interesting molecule of approximately 570,000 daltons. The molecule consists of seven identical chains that are associated by disulfide bonds at their carboxy termini, forming a central "core" (34). The amino terminal portions of the seven chains form tentacular arms which radiate from the core and bear the C4b binding domains, which are homologous to the binding domains of a broad family of proteins that interact with C3b and C4b (see later discussion). C4BP binds to C4b (not C4) and exerts two distinct regulatory actions. The intrinsic dissociation rate of the C4b2a complex is increased, thus shortening the survival of any given classical C3 convertase enzyme (35). In addition, C4b bound by C4BP becomes susceptible to proteolysis by factor I, also known as C3b/C4b inactivator. Factor I cleaves the α' chain of C4b in two places, releasing a four-chain complex termed C4c and leaving a small portion, C4d, covalently bound to the original acceptor surface (36). C4d can no longer support complement convertase activity. Factor I cleavage of C4b on surfaces can proceed without C4BP, albeit slowly, but C4BP is requisite in the fluid phase.

THE ALTERNATIVE PATHWAY OF COMPLEMENT ACTIVATION

History

An alternative pathway for complement activation was described in 1954 by Louis Pillemer and his co-workers at Case Western Reserve University during their investigations of the inactivation of C3 and late-acting complement components by yeast cell walls. They demonstrated that an insoluble yeast cell wall preparation, zymosan, could completely consume C3 during a 37°C incubation in serum without affecting C1, C4, or C2 titers (37). This inactivation had the characteristics of an enzymatic reaction and required factors that could be removed from serum by preincubation with zymosan at 17°C. These factors differed from antibodies in that their absorption from serum required magnesium ions, temperatures above 10°C, a pH of 6.5 to 8.2, and low ionic strength. This led to the suggestion that consumption of C3 occurred by enzymatic activation via a new pathway which was termed the properdin system. Moreover, it was demonstrated that this system played an important role in the serum bactericidal reaction, in viral neutralization, and in the acid lysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria. Unfortunately, subsequent recognition that natural antibody to zymosan is present in normal serum led to the belief that Pillemer's data reflected only the activation of the classical pathway

by preexisting antibody (38). Thus the fundamental discoveries of Pillemer and his colleagues were largely ignored until the alternative pathway was "rediscovered" a decade later.

There are six normal serum proteins thought to be important in the initiation and control of alternative pathway activation. These are factor B, factor D, properdin, factor H (81H), factor I (C3b/C4b inactivator), and C3 itself (39).

Factor D

Factor D is a 25,000-dalton, single-chain glycoprotein, with γ -globulin electrophoretic mobility when purified but α -globulin electrophoretic mobility in serum (40). It is a trace protein in serum (1 to 2 $\mu\text{g}/\text{ml}$), and its low concentration may make it rate limiting in assembly of the alternative pathway C3 convertase, C3bBb. No evidence of cleavage of factor D has been found and it is presumed to circulate in the active state. It has sequence homology with other serine proteases but does not hydrolyze synthetic esters and is relatively resistant to the archetypal serine protease inhibitor diisopropylfluorophosphate (DFP) (41). It has no activity on its natural substrate, factor B, until the latter is bound by C3b. Thus it is most likely that factor D is able to express its proteolytic activity only after exposure of a cryptic site on factor B by the C3b interaction.

Factor B

Factor B is a single-chain, 93,000-dalton, β -globulin zymogen serine protease. In the presence of Mg^{2+} , factor B forms a stoichiometric complex with C3b with a molar ratio of 1:1 (42). On cleavage by factor D, two fragments are formed. The smaller 30,000-dalton fragment, Ba, is released, while the larger 63,000-dalton fragment, Bb, remains associated with C3b. The latter fragment contains the protease activity (43). The complex C3bBb is termed the alternative pathway C3 convertase. This complex is quite labile and Bb dissociates spontaneously from C3b under physiologic conditions. Ba and Bb are reported to have opposing regulatory effects on B lymphocyte function and Bb is reported to augment the spreading of phagocytic cells on a surface.

Properdin

Properdin (from the Latin *perdere*, to destroy), the protein through which the alternative pathway was discovered, is a γ -globulin of approximately 143,000 to 156,000 daltons, consisting of three apparently identical subunits held together noncovalently (44). Its serum concentration is about 25 $\mu\text{g}/\text{ml}$. There are two forms of properdin, native (nP) and activated (P), which apparently differ from each other only by a small conformational change (45). Native properdin can bind to the assembled alternative

pathway C3 convertase (C3bBb) but cannot bind to C3b alone. Its function in this circumstance is to reduce the rate of decay of the convertase and thus promote alternative pathway activation. Activated properdin can, in addition, bind to C3b on particles or in the fluid phase in the absence of factor B and then promote the assembly of C3bBb. Factors regulating the conversion of nP to P are largely unknown, and some authors have questioned whether two distinct forms truly exist. Spontaneous conversion of nP to P in purified protein preparations has been observed to occur, and P is the form of the molecule eluted from alternative pathway activators after incubation in serum. On the other hand, P does not revert to nP spontaneously but is reported to do so after incubation with the denaturant guanidine.

Factor H

Factor H (formerly known as 81H) is a 150,000-dalton, single-chain peptide of β electrophoretic mobility. Its serum concentration is about 500 $\mu\text{g}/\text{ml}$ (46). Gel filtration studies indicate that factor H may circulate as a dimer under physiologic conditions. Unlike the previously described proteins, factors B and D and properdin, which are important in the assembly of the alternative pathway C3 convertase, factor H functions to downregulate C3-cleaving convertase activity. This is accomplished by competition with both B and Bb for C3b binding, thereby inhibiting convertase formation and accelerating decay of existing convertase complexes (47). Factor H is also a necessary cofactor for the inactivation of C3b by factor I (48). The binding of H to C3b does not require cations but is enhanced by low ionic strength. Factor H can bind to C3b on surfaces or in the fluid phase, although with varying affinities depending on the chemical nature of the environment in which the C3b is found. As explained later, this varying affinity of H for C3b may be the most important determinant of whether or not a particle will activate the alternative pathway.

Factor I (C3b/C4b Inactivator)

Factor I is a 90,000-dalton, β -globulin glycoprotein composed of two chains of 50,000 and 40,000 daltons held together by disulfide bonds (49). It demonstrates proteolytic activity toward two substrate molecules: C3b and C4b. In the presence of the appropriate cofactors, factor I effects two sequential cleavages of C4b and three cleavages of C3b (50). These result in defined breakdown products with altered activity (see later discussion). The active site is on the 40,000-dalton light chain and is not inhibitable by DFP, soybean trypsin inhibitor, tosyl-L-lysine chloromethyl ketone (TLCK), benzamide, or phenylmethyl sulfonyl fluoride (PMSF). Nonetheless, sequence analysis has demonstrated a high degree of homology between factor I and known serine proteases. Thus the activity of this enzyme is probably controlled by a requirement for configurational changes in its substrates (C3b or

C4b) mediated by essential cofactors (factor H or C4-binding protein), before exposure of the site for enzymatic cleavage. This is analogous to the mechanism of enzymatic activity of factor D on factor B.

C3

C3, because of its central role in both classical and alternative pathway activation and because of its major role in the host defense process, has been the focal point of much research on complement activation. Human C3 is a 195,000-dalton glycoprotein of β electrophoretic mobility. It has two chains, one of 120,000 daltons (α chain) and the other of 75,000 daltons (β -chain) with carbohydrate present on both chains (51). Serum concentration is 1 to 2 mg/ml. C3, like C4, is synthesized as a single-chain precursor molecule (proC3) in which the β chain occupies the amino terminus. Excision of a series of four arginine residues yields the mature molecule.

Within the α chain resides an unusual thioester bond between a cysteinyl residue and a glutamic acid residue, separated by two intervening amino acids. This unstable bond, thought to be buried in a hydrophobic pocket, is responsible for the covalent binding reactions of C3 (as well as C4 and α_2 -macroglobulin, which share this feature) (52).

When C3 is cleaved by either the classical pathway C3 convertase (C4b2a) or the alternative pathway C3 convertase (C3bBb), the α chain is divided into two unequal fragments, the larger of which remains covalently linked via disulfide bonds to the β chain. This molecule, designated C3b, has a MW of 185,000 daltons (53). The smaller fragment, C3a, represents the amino terminal 77 amino acids of the α chain and has diverse effects on cells with receptors for this peptide, including lymphocytes, mast cells, and endothelial cells (see later discussion). On cleavage of C3 to C3b, the molecule undergoes a complex rearrangement of tertiary structure, which exposes the internal thioester bond in the α chain (52,54). On exposure, the thioester bond can be broken by reaction with appropriate aldehydes, carboxyl groups, nitrogen nucleophiles, or by water itself. The result is a new covalent bond between C3b and an electron-donating group. On particles such as erythrocytes or zymosan, the formation of ester bonds seems to be favored (55), whereas proteinaceous immune complexes form amide, as well as ester, bonds with C3b. Since water can hydrolyze the thioester and is generally present in vast molar excess over all other potential electron donors, the process of C3b binding to surfaces or soluble immune complexes is inefficient. In general, many molecules of C3b will incorporate H₂O into the reactive bond for each molecule that is able to bond covalently to the complement-activating surface. Nonetheless, recent experiments in which C3 was cleaved to C3b in the presence of small molecules, such as amino acids and simple sugars, have demonstrated that some of these are preferential acceptors of the C3b molecule. In particular, glycerol, threonine, and raffinose are highly efficient at forming covalent bonds

with C3b even in the presence of a large excess of H₂O (56). Immunoglobulin G also appears to be an efficient C3 acceptor (see later discussion).

C3b, after formation by either the classical or alternative pathway C3 convertase, is susceptible to further cleavage, which proceeds in several steps (see Fig. 3) (57,58). The first cleavage is made by factor I with H as an essential cofactor for fluid-phase cleavage and an accelerator for cleavage on surfaces. A second factor I-mediated α -chain cleavage occurs in rapid succession with the release of a 3,000-dalton fragment. The resultant three-chain molecule is termed iC3b. It can no longer function in the alternative pathway C3 convertase (or the C5 convertase of either pathway). The iC3b molecule has 68,000- and 43,000-dalton chains derived from the α' chain of C3b and the unchanged 75,000-dalton β chain of the original molecule. Surface-bound iC3b undergoes a third cleavage at a site within the 68,000-dalton chain, which releases a three-chain, 145,000-dalton molecule termed C3c and leaves a 41,000-dalton fragment termed C3dg bound to the surface. This cleavage is carried out by factor I, but factor H does not appear to be a physiologically relevant cofactor (58). Instead the cell membrane C3b receptor, called CR1, appears to serve as cofactor for this step (see later discussion). A variety of enzymes, including trypsin, plasmin, and neutrophil elastase, can remove a 10,000-dalton amino terminal portion from C3dg to yield C3d. These same enzymes can generate C3d directly from iC3b. The breakdown of iC3b in the fluid phase appears to follow the same steps but proceeds very much more slowly and is less well characterized.

ALTERNATIVE PATHWAY ACTIVATION AND CONTROL

When C3b is generated in the course of classical pathway activation, it may serve as an initiator of the alternative pathway by providing a binding site for factor B. The binding requires Mg²⁺. Binding to C3b is presumed to expose a cryptic cleavage site in the factor B molecule, which is the substrate for the protease, factor D. B_a is released, and the resulting bimolecular complex, C3bBb, acts as the C3 convertase of the alternative pathway (59). The active enzymatic site is on Bb, but C3b is essential. C3b in the alternative pathway convertase is able to bind additional native C3 and in so doing makes the cleavage site in the C3 molecule available to the enzymatic activity of Bb. In this way, C3b in the alternative pathway C3 convertase is analogous to C4b of the classical pathway C3 convertase and Bb is analogous to C2a. Factor D is not incorporated into the C3 convertase and may be reutilized. Enzymatic activity is controlled by several mechanisms. Factor H may displace factor Bb from the alternative pathway C3 convertase and also functions as a cofactor for factor I cleavage of C3b, terminating its ability to function in the convertase (60). In addition, the complex enzyme C3bBb has a tendency to dissociate even in the absence of regulatory proteins, with a *T*_{1/2} of about 5 min at 30°C. Properdin counterbalances these controls

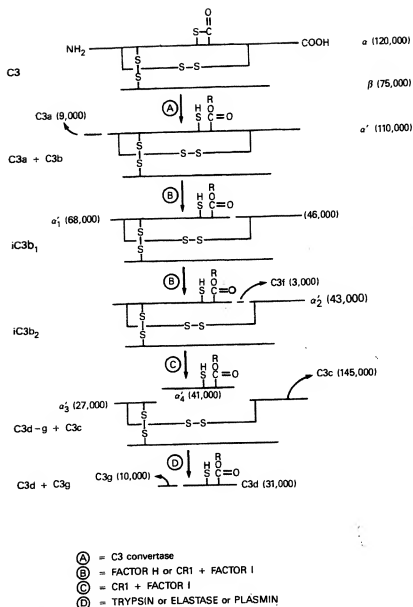


FIG. 3. C3 cleavage and the subsequent stepwise breakdown of C3b. Fragment molecular weights and enzymes responsible for the various cleavages are designated. Recent studies suggest that gp45-70 (membrane cofactor protein) is also a relevant cofactor for step B.

by decreasing the rate of the dissociation of C3bBb and therefore stabilizes enzymatic activity (59). Factors H and I are absolutely required to prevent spontaneous fluid-phase assembly of the alternative pathway C3 convertase and the subsequent consumption of alternative pathway components through unregulated activity of factor D and C3bBb. This has been shown *in vitro* using the purified proteins of the alternative pathway and has also been confirmed *in vivo*, since patients genetically deficient in factor I or H have very low C3 levels and circulating C3 fragments (61). When additional C3b is formed by the action of C3bBb, some portion of the new C3b may join the initial complex to form (C3b)₂Bb. The second C3b provides a binding site for C5 and allows the new complex enzyme to function as the alternative pathway C5 convertase. Kinoshita et al. have very recently demonstrated that the

second C3b responsible for C5-binding activity is actually covalently attached to the initial membrane-bound C3b residue.

Whenever C3b is created by classical complement pathway activation, it may serve as the nidus for formation of an alternative pathway C3 convertase. The alternative pathway convertase can deposit more C3b on the target surface, which can in turn form another alternative pathway convertase site. In this situation, the alternative pathway serves as an amplification mechanism for complement activation and is often referred to as the "amplification pathway."

The initial event of alternative pathway activation in the absence of classic pathway activation is less clear. The most likely sequence of events has been outlined by Pangburn and Muller-Eberhard (59,62). They showed that

the thioester bond in native C3 is not completely stable but subject to slow hydrolysis even in the absence of cleavage of the C3 molecule to C3a and C3b. C3 bearing a hydrolyzed thioester is termed C3(H₂O) and has a number of properties of C3b, including the ability to initiate the alternative pathway by interacting with factor B, the ability to interact with factors H and I, and the capacity to serve as a ligand for cellular C3b receptors (63). Spontaneous hydrolysis of C3 with formation of an alternative pathway C3 convertase leads to a continuous low-level cleavage of C3 in serum called C3 "tickover." This baseline C3 turnover occurs at a slow rate because it is controlled by factors H and I. In the presence of alternative pathway-activating surfaces, such as rabbit erythrocytes, zymosan, or lipopolysaccharide, the tickover process is thought to provide the initial C3b, which binds to these surfaces to begin formation of the surface-bound alternative pathway C3 convertase.

Clearly, not all surfaces support the assembly of an alternative pathway C3 convertase whenever C3b by chance is bound to them through serum C3 tickover. This implies that inherent in the activation of the alternative pathway is the ability to discriminate among surfaces to which C3b binds. A further implication, since normal self-antigens do not activate the alternative pathway, is that the alternative pathway represents a primitive mechanism for distinguishing self-antigens from foreign antigens, such as yeast cell walls (zymosan) or bacterial cell walls or lipopolysaccharide. The molecular nature of this discriminatory ability is partially understood. Activators of the alternative pathway provide a "protected site" for C3b binding, that is, one in which the net effect is to favor factor B binding to C3b over factor H binding (59). In this setting, the alternative pathway C3 convertase will form and be able to cleave more C3 without the usual inhibition by factor H. With rare exception, the property of alternative pathway-activating potential correlates with a reduced affinity of surface-bound C3b for factor H. This has been best demonstrated in investigations of the role of cell surface sialic acids in control of alternative pathway activation (64,65). Although sheep erythrocytes do not activate the human alternative pathway, sheep cells from which sialic acid residues have been removed will activate the alternative pathway, and the extent of activation is proportional to the amount of sialic acid removed. When the mechanism for this was investigated, it was discovered that, although C3b molecules on normal and desialated sheep erythrocytes demonstrated equal affinity for factor B, the affinity of C3b for factor H on fully sialated cells was 10- to 20-fold greater than on desialated cells (66). Thus, in this case, cell surface sialic acid, by increasing the binding of H to C3b, prevents the formation of an effective alternative pathway C3 convertase. A similar mechanism for control of alternative pathway activation was demonstrated for heparin that had been linked to zymosan particles. Interestingly, removal of sialic acid from human erythrocytes does not lead to alternative pathway activation. Thus additional control mechanisms are operative on human cells, some of which are discussed subsequently.

Role of Immunoglobulin

No obligate role for antibody exists in the activation of the alternative pathway, but immunoglobulin can exert a strong modulating influence on this system (67). Aggregated human myeloma proteins of the IgG, IgM, IgA, and IgE classes are capable of activating the alternative pathway (68). Soluble and particulate antigen-antibody complexes formed of IgG from several mammalian species and intrinsically nonactivating antigens have also been shown to activate the alternative pathway, and this can be shown to occur without classical pathway participation. Additionally, sensitization with specific IgG significantly augments the rate and/or extent of alternative pathway activation by intrinsically activating surfaces. This effect is independent of the Fc portion of the IgG molecule and has been observed to occur with both F(ab')₂ and Fab fragments.

The mechanisms whereby IgG influences the alternative pathway are several. IgG is an excellent acceptor of nascent C3b, the majority of which binds to a site in the heavy chain and probably within the Fd fragment (69). Thus antibody may enhance the deposition of C3b and the rate of formation of new C3 and C5 convertases by providing additional acceptor sites on a sensitized surface. Alternatively, antibody may function by masking sialic acid residues that would normally inhibit alternative pathway activation, as has been shown using antibodies to the capsule of group B streptococci (70). Finally, data suggest that C3b covalently bound to IgG is less susceptible to inactivation than free C3b or C3b bound to a non-immunoglobulin acceptor protein (71). This appears to be due to a reduced affinity of factor H for C3b bound to IgG. The relevance of this finding to surface-bound C3b remains to be established, but it may in part explain the observed relative resistance of C3b bound to IgG-bearing soluble antigen-antibody complexes to the action of factors H and I (72).

An interesting autoantibody has been shown to influence alternative pathway activation in a manner quite different from those previously discussed. This antibody, termed C3 nephritic factor (C3NeF), was discovered in the sera of patients with membranoproliferative hypocomplementemic glomerulonephritis and was subsequently shown to be present in some individuals with partial lipodystrophy (73). C3NeF is an IgG antibody specific for the alternative pathway C3 convertase, C3bBb. The complex C3bBbC3NeF is highly resistant to factor H-mediated dissociation and is thus capable of promoting unregulated C3 consumption by the alternative pathway.

Agents that Modify Alternative Pathway Activation

There are a number of substances that have proved to be useful modulators of alternative pathway activation in the laboratory. These can be divided into two groups, those that promote C3 cleavage and those that inhibit C3 cleavage.

One of the most widely used of such reagents is a 140,000-dalton protein from cobra venom that is functionally analogous to C3b. This three-chain molecule, termed cobra venom factor (CVF), is derived from the cobra's C3 (74). It can bind factor B, permit its cleavage by D, and form an alternative pathway C3 convertase. Unlike C3b, CVF is not inactivated by H and I and therefore forms an unregulatable C3-cleaving enzyme. The unregulated convertase is able to cleave C3 and factor B in the fluid phase until both proteins are essentially completely consumed from serum. This activity of CVF has been used by investigators to deplete C3 both *in vivo* and *in vitro* and as a way of obtaining C3b. The extent of consumption of terminal complement components following CVF activation of the alternative pathway depends on the source of CVF. Cobra venom factor isolated from the venom of *Naja naja kaouthia* forms not only an unregulated C3 convertase but also an effective C5 convertase. C5 cleavage is followed by consumption of all the terminal components. Cobra venom factor from *Naja haje* venom, on the other hand, is incapable of C5 cleavage; thus its use leads to consumption of B and C3 but leaves terminal component levels unaffected. The molecular mechanism for these differences in CVF molecules has not yet been elucidated.

Other agents that promote alternative pathway activation include suramin, a drug used in the treatment of African trypanosomiasis, and K-76 monocarboxylic acid, a product of the fungus *Stachybotrys complementi* K-76 (75,76). Both inhibit cleavage of C3b by factors H and I, albeit by different mechanisms.

Agents that inhibit alternative pathway activity include heparin and gold sodium thiomalate, both of which appear to increase the affinity of C3b for factor H (77,78). Fluid-phase heparin also appears to be capable of "masking" a portion of surface-bound C3b residues and preventing their interaction with factor B. Complestatin, a product of *Sreptomyces lavendulae*, inhibits alternative pathway activation by combining with factor B in a manner that blocks its interaction with C3b. Recently, a macromolecular product of *Aspergillus fumigatus* has been identified which inhibits alternative pathway activation by as yet unclear mechanisms (79). In addition, a membrane glycoprotein encoded by herpes simplex type 1 virus has been shown to modulate both the alternative pathway C3 convertase and C3-C5 interactions (80). This may result in enhanced resistance of the virus to neutralization in serum (81).

THE C1 BYPASS PATHWAY

There is a third pathway of complement activation, discovered by the use of C4-deficient guinea pig serum. In this pathway, although no classical pathway C3 convertase is formed, C1 activation is required for activation of the complement cascade. Therefore this pathway has been called the C1 bypass pathway (82). Although the mechanism for this effect is unknown, one possibility is that activated C1 replaces D (albeit inefficiently) in con-

centrated C4-deficient serum. Initiation of complement activation via this pathway requires a large amount of antibody. The molecular natures of the C3 and C5 convertases in this pathway are unknown. Recently, this pathway has been shown to be responsible for the lysis of *Giardia lamblia* as well as several bacterial species (83).

TERMINAL COMPLEMENT COMPONENTS AND THE LYTIC MECHANISM

A detailed understanding of the molecular interactions of the terminal components C5 to C9 has developed over the last two decades. It is known that biologic activity of C5 requires cleavage of the C5 molecule into two fragments, C5a and C5b, by an enzyme derived from the C3 convertase. The C5a fragment has potent independent biologic activity which is considered later. The C5b portion combines sequentially with C6, C7, C8, and C9 to form a macromolecular complex that is capable of damaging biologic and artificial membranes and causing cell lysis by the creation of a hydrophilic transmembrane pore or channel (84,85). In addition, a phenomenon known as reactive lysis has been described, in which the isolated metastable C5b6 complex can be used to initiate formation of the terminal complex on surfaces without a concomitant requirement for earlier components. This reaction has provided a useful tool for identifying potentiators and inhibitors of terminal complex formation (86). The most intensive areas of investigation have focused on the exact molecular composition of the C5b-9 complex, the biochemical nature of attachment of this complex to membranes, and the functional and biochemical characteristics of the lytic complement lesion. After much debate, most workers in the field agree that complement lysis is due to the formation of a stable, hydrophilic transmembrane channel, as predicted by the "doughnut model" of Mayer et al. (85). There is now a general consensus that complement lysis of cells results because the terminal complex creates membrane channels that are large enough to permit exchange of small molecules and ions but too small to permit release of macromolecular cytoplasmic constituents. Due to the Donnan effect, water enters the cells through such channels causing the cell to swell and burst.

A large body of literature now substantiates that during formation of C5b-9, the complex inserts into the lipid bilayer of membranes. Evidence includes the observations that (a) hydrophobic domains are exposed within the forming C5b-9 complex, (b) phospholipids are released from target membranes during complement attack, (c) the attached C5b-9 complex cannot be eluted from membranes by ionic manipulations or aqueous-phase proteases but instead requires detergent for extraction, (d) conductivity changes are detected across planar lipid bilayers attacked by the terminal complex, and (e) constituents of C5b-9 can be labeled by membrane probes that localize exclusively within the hydrophobic core of lipid bilayers.

Initiation of C5b-9 Formation: C5 and the C5 Convertase

Initiation of membrane attack complex (MAC) formation requires cleavage of C5. C5 is a β -globulin glycopro-

tein comprising a 115,000-dalton α -chain disulfide linked to a 75,000-dalton β chain. Considerable sequence and structural similarity exists between C3, C4, and C5, but the latter lacks a thioester and does not form covalent bonds with target surfaces (51). Physiologic cleavage of C5 produces a 185,000-dalton C5b fragment and an 11,000-dalton C5a peptide. In cellular systems, the hemolytically active C5b remains cell associated whereas the smaller C5a fragment is released into the fluid phase. Recent data suggest that release of C5a is accelerated by interactions of C6 and C7 with newly cleaved C5 (87).

C5 is cleaved by enzymes formed during both alternative and classical pathway activation. The C5 convertase enzyme of the classical pathway is the C4b2a3b complex. Enzymatic activity resides in the C2a molecule within the complex. The enzymatic site for C5 cleavage by the alternative pathway convertase, (C3b)₂Bb, is contained within Bb. In both the classical and alternative pathway C3 convertases, physical decay of the enzymatic subunit from the complex results in loss of C5 convertase activity. Cleavage of C5 by either convertase results in identical fragmentation of the molecule. In each convertase, C3b binds native C5, exposing the site of enzymatic cleavage. Current data indicate that C3b covalently attached to C4b is the relevant C5-binding site in the classical pathway enzyme (88). Recent evidence suggests that there are two molecular forms of cell-bound C3b in the alternative pathway C5 convertase. One molecule appears to function like C4b in the classical pathway convertase, and one molecule is analogous to C3b of the classical pathway enzyme. The C3b serving as a C4b analog binds Bb, maintaining it in an enzymatically active configuration. Fluid-phase C3b can also function as a binding site for C5, with subsequent cleavage by a fluid-phase enzyme complex. It is likely that the major requirement for effective cell surface C5 binding and cleavage is a high local concentration of C3b.

There is a linear relationship between the number of cell-bound C5 convertase complexes and subsequent C5 uptake. However, under conditions in which all C5 hemolytic activity is consumed, only a small percentage of C5 binds to the activating surface. Moreover, cell-bound C5b rapidly loses hemolytic activity despite a substantially slower rate of loss of C5 antigen from the cell surface. Thus it appears that cell-bound C5b undergoes a rapid conformational change that renders it hemolytically inactive. The nature of the change is still unclear. As discussed in more detail later, the presence of C6 and C7 increases the uptake of C5 onto membrane surfaces and stabilizes the hemolytic activity of the cell-bound C5b.

Noncomplement-Mediated C5 Cleavage

A large number of noncomplement proteases produce cleavage of C5 to yield biologically active peptides. A detailed review of these reactions is beyond the scope of this chapter but has been well summarized (89). Briefly, trypsin, plasmin, polymorphonuclear leukocyte proteases such as elastase and cathepsin G, macrophage and platelet

proteases, and bacterial enzymes have been shown to cleave native C5 into biologically active peptides. It is now clear that the fragments produced by trypsin cleavage of C5 are not equivalent to the fragments formed when C5 has been cleaved by a C5 convertase. Specifically, C5a is not produced, although a C5a-like biologic activity is generated. The anaphylatoxic and chemotactic activity of trypsin-cleaved C5 resides in fragments that remain disulfide linked to the high-MW, C5b-like polypeptide.

C5b6 and Reactive Lysis

C6, the next molecule in the cascade, is a β_2 -globulin with a molecular weight reported between 105,000 and 128,000 daltons. Its structure is stabilized by numerous intrachain disulfide bonds (90). C5b and C6 can form a stable complex in serum that retains its ability to interact with cell membranes over time. With the addition of C7, C8, and C9, the C5b6 complex can lyse unsensitized erythrocytes and certain other cells in the absence of the antecedent components, a phenomenon known as reactive lysis (86). The conformational alterations in C5b which enable interactions with C6 are very short-lived, and C5 cleavage must occur in the presence of C6 for effective C5b6 complex formation to occur.

The biochemical characteristics of the C5b6 molecule have been defined (91). The complex has an *S* rate of 10.4 to 11.5, a MW of 328,000 to 330,000, and in its hemolytically active form contains a single molecule each of C5b and C6. The electrophoretic mobility of the isolated complex is indistinguishable from that of C5 (β_1) but faster than that of C6 (β_2). The C5b6 complex expresses a new antigenic determinant (neoantigen) that is not present on either C5 or C6 and is presumably related to conformational changes in the C5b and C6 molecules during complex formation (92).

C7, Formation of C5b67, and Inhibitors of C5b67

C7 has physical characteristics very similar to C6 and exhibits extremely high affinity for the C5b6 complex. It is reported to have 23 to 30% sequence homology with the later-acting components C8 and C9 (93). Attachment of C7 to newly formed C5b6 occurs very rapidly via hydrophobic interactions and results in the formation of a labile fluid-phase complex that aggregates and loses activity if not attached to acceptor surfaces. The C5b67 complex expresses a unique second neoantigen that differs from that of C5b6. This trimolecular complex represents the first product of complement activation to stably insert into the lipid bilayer of target membranes (94). The activity of the complex is very evanescent at physiologic temperatures and membrane insertion is inefficient (less than 1%) unless the target is sensitized with C3b. The presumptive mechanism of the C3b effect is binding of C5b to C3b, thereby promoting C5b67 formation close to the target surface and increasing the likelihood of subsequent hydrophobic interactions (95).

A number of substances have been defined that act on C5b67 reversibly in the fluid phase to prevent attachment to bystander erythrocytes during the short time that the hydrophobic binding site of C5b67 is available. One class of inhibitors includes the low-density lipoproteins and serum S protein, each of which presumably binds to the nascent hydrophobic membrane-binding site on C5b67 and blocks subsequent interaction of the complex with target membranes. A second class of inhibitors includes various polyanions such as heparin, dextran sulfate, and DNA. On the other hand, histones and polycations enhance the lytic activity of C5b67 for bystander erythrocytes. The mechanisms by which these inhibitors and potentiators act are still unclear, but presumably they function by modifying ionic or charge interactions of C5b6 or C5b67 with the target membrane. It has also been suggested that C8 limits the activity of C5b67 by preventing the attachment of the fluid-phase complexes to target membranes. Thus C8 serves a dual role in complement-mediated cytotoxicity and represents a point of internal regulation in the late steps of the complement attack sequence.

C8

C8 is a three-chain γ -globulin with α and β subunits of 64,000 daltons and a smaller γ chain of 22,000 daltons. The α and β chains differ in sequence despite their identical molecular weights (93,96). The α and γ chains form a disulfide-linked heterodimer which associates noncovalently with β chain in serum, where the complex exists as an equilibrium mixture. A single molecule of C8 can interact via its β chain with each C5b67 complex and this interaction has an extremely high affinity constant similar to that of C7 uptake by C5b6. When C8 binds to C5b67 on a membrane, the C8 γ chain is inserted into the hydrophobic regions of the phospholipid bilayer as assessed by labeling with hydrophobic photoreactive probes and inaccessibility to proteases. Insertion of C8 α chain through the lipid bilayer constitutes a lesion sufficient to initiate erythrocyte lysis, leakage of radiolabeled solutes, and significant increases in membrane conductivity (84). Target lysis by C5b-8 complexes is slow, however, and the hydrophilic lesions which are apparently formed are both small (0.4 to 3 nm) and unstable. Dramatic enhancement of lytic efficiency occurs on addition of the next component, C9.

C9

C9 is a single-chain α -globulin of 71,000 daltons. It is composed of two distinct domains. The smaller, C9a, is rich in acidic residues and hydrophilic. The larger domain of 37,000 daltons, C9b, is enriched for hydrophobic residues, although the deduced amino acid sequence does not include a single extended hydrophobic region similar to those of typical transmembrane proteins (94). Both C9a and C9b contain multiple intrachain disulfides which ap-

parently do not cross domain boundaries. Antibodies elicited by denatured C9b are cross-reactive with C6, C7, and C8 α chain, as well as the pore-forming protein perforin, isolated from cytotoxic lymphocyte granules. Thus these proteins may have similar structures responsible for their hydrophobic interactions (94).

C9 has the capacity to polymerize, and purified C9 can be induced to do so by prolonged 37°C incubation. Polymerization is accompanied by elongation of the molecule, display of a distinctive neoantigen, an increase in surface hydrophobicity, and the appearance of free sulfhydryl groups. These latter participate in interchain disulfide bond formation, to produce C9 dimers. Polymerized (or "poly") C9 forms highly ordered tubular structures. These have a length of approximately 16 nm and an internal diameter of approximately 10 nm. One end bears a thickened annulus, while the other is hydrophobic and readily inserts into phospholipid bilayers. The average molecular weight of poly C9 is 1.1×10^6 daltons, but the complexes are heterogeneous and may contain 11 to 19 C9 monomers, although the more common forms contain 14 to 16 (84,94,97).

CHARACTERISTICS AND COMPOSITION OF THE FLUID-PHASE AND MEMBRANE-BOUND MEMBRANE ATTACK COMPLEX (MAC)

There is still controversy surrounding the details of complement lysis (84,94,97). In most situations the attack mechanism of complement requires the participation of C5b, C6, C7, C8, and C9 as a macromolecular complex with a molar composition of 1C5b:1C6:1C7:1C8:3-6C9. A stable fluid-phase 22.5S complex with a MW of 1.04×10^6 with the electrophoretic mobility of an α -globulin and containing C5b-9 can be demonstrated after incubation of serum with alternative or classical pathway activators. The complex assembled in the fluid phase has no activity but is able to inhibit lysis of EAC₁₋₈ by C9, presumably because the fluid-phase complex, with a molar ratio of 1C5b:1C6:1C7:1C8:3C9, binds additional C9. Such complexes also contain three molecules of an additional protein, S protein (97). S protein circulates as a normal constituent of serum at a concentration of 600 μ g/ml. This protein binds to the nascent hydrophobic binding site of C5b67 during formation of fluid-phase terminal component complex, thereby destroying its lytic activity and preventing its aggregation.

The physical characteristics of the membrane-bound MAC (C5b-9) have been studied following extraction from erythrocyte membranes by nonionic detergents. The complex within the membrane behaves like an intrinsic or integral membrane protein since it cannot be extracted with high ionic strength buffers or EDTA. This observation provided some of the first evidence that the MAC associated with the membrane through hydrophobic interactions. The dimensions of the extracted complex as determined by electron microscopy suggested the presence of monomeric C5b-9 complexes with an estimated MW of 1×10^6 . However, others have since reported that the

membrane-bound form of the terminal complex is a C5b-9 dimer or consists of monomeric, dimeric, and trimeric forms, and the MAC may in fact have a heterogeneous size distribution.

Analysis of both cell-bound and fluid-phase C5b-9 complexes has suggested that they contain a disulfide-linked dimer of C9. Such C9 dimers may serve an important function in cell lysis by facilitating dimer formation of the entire C5b-9 complex, thus enhancing membrane disruption. However, this hypothesis remains to be proved.

Electron microscopic studies of the lytic complement lesion using the negative stain technique demonstrated that the lesion has the appearance of a doughnut, with an annular rim of 15 nm in diameter and a central, electron-dense region of 10 to 11 nm in diameter raised above the membrane (Fig. 4) (98). More detailed examination of the detergent-extracted complex from erythrocyte membranes has led to a model in which the complex contains a cylindrical stalk of 15 to 16 nm in length constituting the portion of the complex that is embedded into the hy-

drophobic core of the membrane. The annulus or torus of the complex projects above the membrane by at least 10 nm (Fig. 4), has an external diameter of 20 to 25 nm, and an internal diameter of 10 to 11 nm for human complement and 8.5 to 9.5 nm for guinea pig complement. When the isolated complex is reincorporated into lipid vesicles, the cylindrical axis of the complex is oriented perpendicularly to the membrane, and the annulus is located external to the vesicle (Fig. 4).

The interpretation of the electron microscopic appearance of the terminal complex has been modified in light of data developed by Podack and Tschopp (review in ref. 97). Based on the remarkable similarities between poly C9 and extracted MAC formed in C9 excess, these investigators have proposed that the membrane-bound MAC is composed largely of poly C9, with more limited participation of C5b678. Studies with membrane-restricted hydrophobic photoreactive probes are consistent with this view, since addition of C9 to membranes bearing C5b678 appears to reduce the exposure of the C5b678 to

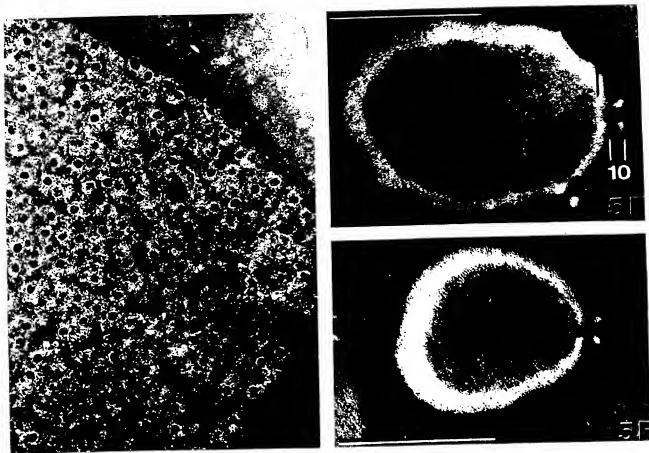


FIG. 4. Electron micrographs of the C5b-9 or membrane attack complex lesions. **Left:** A negatively stained erythrocyte membrane after complement attack, demonstrating innumerable discrete "holes." (Courtesy of Dr. R. Dourmashkin.) **Right:** A single complex penetrating a liposome. The long axis of the complex is perpendicular to the lipid surface and the hydrophobic domain is inserted 4 to 5 nm through the lipid milieu. The complex projects into the aqueous phase approximately 10 nm and displays a thickened outer annulus. (From Bhakdi and Tranum-Jensen, ref. 99, with permission.)

the hydrophobic milieu. C5b678 complexes in membranes bind C9 with extremely high affinity and with stoichiometries consistent with poly C9 formation (12–15C9:1C8). The resultant complexes appear to contain C5b and the C8 β chain in a rodlike 17-nm projection, while C6, C7, C8 α - γ , and C9 are associated with the tubular lesion itself.

The formation of tubular poly C9 is not, however, a *sine qua non* for expression of the lytic properties of C5b-9 complexes. Functional transmembrane lesions are formed by complexes bearing numbers of C9 molecules well below that required for poly C9 formation, and the functional diameter of such channels is roughly proportional to the number of C9 molecules in the complexes (100). Furthermore, thrombin-cleaved C9, which cannot form poly C9, remains capable of supporting complement-mediated cytolysis. Thus while poly C9 formation may have an important role in the genesis of the classical MAC structure, the nature of the minimal effective lytic C5b-9 complex remains to be resolved.

CELLULAR COMPLEMENT RECEPTORS

Many of the biologic functions of complement are mediated by interaction of complement cleavage fragments with specific cell membrane receptors. The interaction of these complement component products with their receptors triggers a series of complex biochemical responses within the cells (101–103). Some of these fragments, such as C3b, iC3b, and C3dg, are bound to the site of complement activation on a target particle. The interactions of these target-bound components with their cellular receptors is thought to trigger a number of specific responses such as phagocytosis by neutrophils and macrophages, or B lymphocyte activation. Complement activation also generates several smaller peptides, C3a, C4a, and C5a, that are released into serum or extracellular fluid. This group of three polypeptides constitutes the complement-derived anaphylatoxins.

Anaphylatoxins and Their Receptors

The binding of these released polypeptides to specific cell surface receptors leads to a series of cellular responses important to the initiation and maintenance of the inflammatory process.

C3a is released on cleavage of C3 by either the classical or the alternative pathway C3 convertase. It is a 9,000-dalton, nonglycosylated protein with a pI of 9.7. C3a constitutes the N terminal 77 amino acids of the C3 α chain (104,105). Receptors for C3a have been identified on mast cells and basophils, smooth muscle cells, lymphocytes, and perhaps platelets. Engagement of the C3a receptors on mast cells or basophils induces degranulation, with release of histamine and other mediators of anaphylaxis; thus the name anaphylatoxin. Binding of C3a to tissue preparations induces contraction of smooth muscle cells. Whether smooth muscle contraction is mediated directly, or secondarily by histamine, is unclear. Although ileal

contraction in response to C3a can be blocked by antihistamines, uterine contraction cannot (105,106). *In vitro* studies have also shown that C3a induces the secretion of mucus by goblet cells, another important feature of allergic and anaphylatoxic responses.

C5a, the "classic" complement anaphylatoxin, is an 11,000-dalton protein that represents the N terminal 74 amino acids of the C5 α chain. Approximately 25% of its MW is contributed by a single asparagine-linked oligosaccharide (104,105). Interestingly, the human peptide is heavily glycosylated but C5a from certain other species, such as the pig, has little or no carbohydrate. The function of the carbohydrate is unknown. In many systems removal of the carbohydrate leaves the activity of C5a unaltered. C5a is approximately 200-fold more potent as an anaphylatoxin than is C3a. Part of its anaphylatoxic effect is mediated by direct binding to a specific receptor on basophils and presumably mast cells. Part of its effect is indirect, mediated via binding to neutrophils which presumably then release a mast cell degranulation-inducing substance. C5a and C3a both show tachyphylaxis. Cells stimulated with one of these peptides will respond less well to a second stimulation with the same molecule. They will, however, respond to stimulation by the other anaphylatoxin. This is taken to indicate that each peptide binds to its own distinct and specific receptor. In addition to its anaphylatoxic effects on mast cells and smooth muscle, C5a also has histamine-independent effects on endothelium, causing increased vascular permeability. C5a also induces the directed locomotion (chemotaxis) of neutrophils and monocytes. The chemotactic receptor on these cells for C5a has been well studied (107). Binding of C5a to its receptor has a dissociation constant of 2×10^{-9} M, and there are about 2×10^5 C5a receptors per neutrophil (108). In its ability to promote chemotaxis, C5a is quite different from C3a, which apparently has no effect on leukocyte chemotaxis. C5a has a number of other important effects upon neutrophils, causing increased adhesiveness, aggregation, and adherence to endothelium, and also triggering both degranulation and oxidative burst activity (109).

C3a and C5a have also recently been shown to have important and opposite effects on *in vitro* immunoglobulin production by human lymphocytes. C3a suppresses and C5a enhances immunoglobulin secretion; both apparently act at the level of the T cell, presumably through receptor-mediated mechanisms.

A third peptide generated during complement activation, C4a, has only recently been shown to possess anaphylatoxic activity. It is a nonglycosylated, cationic peptide of 8,650 daltons. It is 100-fold less potent as an anaphylatoxin than C3a and 25,000-fold less potent than C5a. Its potential effects on neutrophils and lymphocytes have not been examined. Interestingly, C4a can elicit cross-tachyphylaxis to C3a, but not C5a, in guinea pig ileum. This suggests that C4a and C3a may act through the same receptor.

All the complement anaphylatoxins are rapidly cleaved in serum by the action of carboxypeptidase N, which removes the carboxy terminal arginine shared by all three molecules (104,105). The loss of the C terminal arginine

destroys the anaphylatoxic activity of all three molecules. However, C5_{ades arg}, although about 10-fold less potent than native C5a, does retain some chemotactic activity. A 60,000-dalton noncomplement serum protein called helper factor has been identified which, on binding to C5_{ades arg}, further increases its chemotactic activity without restoring anaphylatoxin activity (109). This protein may act to distort or obscure the carbohydrate present on C5_{ades arg} since deglycosylated C5_{ades arg} has been shown to be 10-fold better as a chemoattractant than unmodified C5_{ades arg}.

Integral Membrane Proteins that Regulate Complement Activity

One of the most important developments in complement research over the past 20 years has been the recognition of a series of integral membrane proteins present on cellular surfaces that regulate complement activation, degradation, and biologic activity. In many cases these proteins also function as membrane receptors. The binding of ligand to these receptors influences the activity or state of differentiation of the cells on which they reside.

The Receptors for Target-Bound Fragments of C3: CR1, CR2, CR3, and CR4

The best-studied cellular receptors for complement components are those involved in complement enhancement of the phagocytosis of bacteria, yeast, antibody-sensitized erythrocytes, and other particles. It has been shown that receptors for several C3 fragments are of great importance in phagocytosis. The discovery of cellular complement receptors is credited to Nelson in 1950. Although earlier reports are replete with examples of fresh serum promoting binding of particles to phagocytic cells, Nelson showed that neutrophil phagocytosis of *Treponema pallidum* and *Streptococcus pneumoniae* sensitized by antibody and complement was more efficient in the presence of human erythrocytes (110). This erythrocyte enhancement of phagocytosis was thought to occur because complement-coated bacteria were immobilized on the surface of the red cells and thus more easily engulfed. Ultimately, this red cell binding of bacteria was shown to occur because of the interaction of organism-bound C3b with a specific C3b receptor on the erythrocytes, a phenomenon known as immune adherence. All primate erythrocytes possess immune adherence (C3b) receptors; although nonprimate erythrocytes do not have this receptor, a functionally equivalent receptor exists on the platelets of nonprimate vertebrate species. Receptors for C3b have also been demonstrated to exist on B lymphocytes, some T lymphocytes, monocytes, and neutrophils as well as mast cells, eosinophils, basophils, and glomerular podocytes (102). The C3b receptors on all blood cells are antigenically identical but the receptor isolated from granulocytes has a slightly higher molecular weight on gel electrophoresis. The isolated C3b receptor glyco-

protein is found in a series of genetically determined polymorphic forms and varies in size from 190 to 280 kd (see section on molecular biology). Its configuration in the cell membrane is unknown, although some evidence suggests that it exists in the form of a hexamer or pentamer. The C3b receptor, in order to distinguish it from membrane receptors for other C3 fragments, is termed CR1. There are 300 to 1,000 CR1 on each erythrocyte and about 5,000 to 30,000 CR1 per cell for the various leukocytes, as determined by binding of specific anti-receptor monoclonal antibody. Since there are about 1,000 times as many erythrocytes as leukocytes in the blood, this implies that over 90% of circulating CR1 are on erythrocytes. CR1 binds C3b with a much higher affinity than native C3, perhaps as much as 1,000-fold higher (63). Hence, the interaction of a C3b-coated particle or immune complex with the C3b receptor is not blocked by free serum C3, allowing C3b to play an important role in phagocytic processes and the catabolism of immune complexes. CR1 remains in the detergent-soluble fraction, suggesting that it is not associated with cytoskeletal elements. Presumably, in erythrocytes the molecule retains this configuration. On phagocytes, activation of the cell with lymphokines, phorbol esters, or a number of other soluble immunomodulators leads to clustering and ultimately capping of the receptor. It becomes detergent-insoluble, suggesting cytoskeletal attachment (111). Moreover, the receptor is endocytosed. Some data suggest that such endocytosis actually represents cycling of the receptor to an internal pool with subsequent return of the receptor to cell surface (112). If monomer ligand (C3b) is bound to the receptor, it too returns to the cell surface in unaltered form. However, internalized dimeric or multimeric C3b is transferred from the CR1-containing compartment to other compartments within the cell, where it is ultimately catabolized. Evidence obtained with phorbol ester-stimulated cells suggests that CR1 is rapidly phosphorylated and that phosphorylation is reversed after 30 min of incubation with the stimulant. Other stimuli, like the chemotactic peptide f-met-leu-phe, also cause phosphorylation although, unlike phorbol esters, they do not induce ingestion of targets such as C3b-coated red cells.

The binding of a C3b-coated target to CR1 does not itself initiate phagocytosis by a resting phagocyte. A second signal is generally required to initiate the phagocytic process (113). This signal can be provided by a few molecules of IgG bound to the target interacting with IgG Fc receptors on the cell membrane. It can also be provided by various activation signals that appear to take the cell from a resting state to an activated state. Such activation signals can be provided by factors released during chronic infection, fibronectin or laminin acting on mononuclear phagocytes, activators of the phosphoinositide metabolic pathway, and phorbol esters (103).

In addition to its role in phagocytosis, CR1 plays a critical role in the C3 degradative pathway. Upon binding of C3b to CR1, it becomes accessible to the action of factor 1. In this respect it acts like fluid-phase factor H; however, the product of this interaction is not the fragment iC3b

mentioned earlier (see Fig. 3). In contrast, cleavage proceeds beyond the iC3b step to yield a further degradative product, C3dg (102,114). CR1 also facilitates the degradation of C4b by factor I, leading to the formation of a cleavage fragment, C4d, bound to the target surfaces. CR1 is believed to play a critical role in the processing of immune complexes. These appear to arise in the circulation quite commonly, for example, during the course of a viral infection, and are potentially quite toxic. It appears that the binding of C3b to these immune complexes is important in their ultimate removal from the circulation. Upon activation, C3b tends to bind to immunoglobulin (see previous discussion). Once immunoglobulin bound, the C3b tends to resist further degradation by factors H and I (71,114). The C3b will interact with adjacent cells with C3b receptors, and the bulk of CR1 receptors in the circulation are on erythrocytes. Such interaction leads to effective removal of the immune complexes from the plasma by adsorption to the erythrocyte surface, and the complexes can no longer diffuse from the intravascular compartment into tissue sites to induce damage. The C3b-containing immune complexes bound to erythrocytes circulate to the liver where they are stripped off the erythrocytes by macrophages within the hepatic sinusoid (115). The erythrocytes, now free of immune complexes, return to the circulation where they continue to circulate with a normal half-life. Interestingly, they appear to lose some membrane CR1 in this process, and states associated with the presence of high levels of circulating immune complexes are characterized by circulating erythrocytes with decreased numbers of CR1 (116).

CR3, CR4, and CD18 Family of Proteins

The product of C3b cleavage by factors H and I is a three-chain molecule, iC3b (see Fig. 3). There are receptors for iC3b on a variety of cell types including PMNs, monocytes, lymphocytes responsible for antibody-dependent and NK cellular cytotoxic activity, and mast cells (102,103). The iC3b receptor (termed CR3 or Mac-1) is functionally the best characterized of all the complement receptors. It is a two-chain molecule with a 150-kd α chain and a 95-kd β chain. This receptor is part of a family of such proteins, each with a distinct α chain, which all share the same β chain. Other members of this family (CD18) include LFA-1 (lymphocyte function antigen-1) and p150-95, another C3-binding molecule now thought to represent CR4. The function of the β chain appears to be to direct insertion of the complex into the cell membrane (117). A portion of the cellular CR3 content resides within granules in neutrophils and is translocated to the cell surface when the cell is activated. The location of the receptor within the neutrophil has been reported by many workers to be the specific granule. However, a portion of the receptors may reside in another, more poorly defined, granule compartment that also contains the enzyme gelatinase. CR3 is quite specific in terms of ligand binding; in the presence of Ca^{2+} and Mg^{2+} ions, it will bind iC3b but does not recognize C3b. C3dg or C3d are bound weakly. It is re-

ported that the receptor recognizes in part a characteristic arginine, glycine, asparagine (RGD) sequence in the α chain of C3, but that yet another binding site on the molecule is required for stable binding. The receptor is also reported to have conglutinin-like properties, binding to carbohydrate on C3 via a lectinlike interaction. This may represent the additional binding site (103). Like many receptors that recognize the RGD sequence, this receptor is reported to be important in cellular adherence and cells deficient in the receptor have a major adherence defect. A group of children have been reported who lack or have very low levels of the CD18 family of glycoproteins on their cell membranes. The phagocytes of these children ingest particulate targets poorly and also have multiple defects in other adherence-dependent functions. As a result of this deficiency, the children have frequent soft tissue and cutaneous infections with a variety of bacterial pathogens, especially staphylococci and *Pseudomonas aeruginosa* (118). Ligands that interact with proteins of the CD18 family are reported to appear on endothelial cells when these cells are treated with certain lymphokines, suggesting that the release of lymphokines facilitates the attachment of immune effector cells to endothelium, which is the first step in emigration into areas of tissue inflammation. In the phagocytic process itself, CR3 functions much like CR1, requiring a second signal for phagocytosis in the resting cell.

CR2 and Other Complement Peptide Receptors

This 140-kd integral membrane protein recognizes the physiologic C3 product formed upon interaction of CR1 with C3b and factor I, C3dg. Elastase and other tryptic enzymes can cleave C3dg to a further degradative fragment, C3d, which is also recognized by CR2. This integral membrane protein is present on all B lymphocytes and epithelial cells and is reported to be important in providing the B lymphocyte with signals that stimulate cell cycling and differentiation (119). CR2 also serves as the cellular target for the binding of Epstein-Barr virus to B lymphocytes. Its presence on epithelial cells is believed to be important in initial Epstein-Barr virus infections in which epithelial cells of the pharynx are first invaded. Although CR2 is not found on phagocytes, the C3dg or C3d fragments on a particulate target can facilitate binding and ingestion via interactions with CR3 and CR4.

There are receptors for several other complement components that have been identified on various cell types, but their biologic importance is not yet understood. A receptor for factor H has been reported on B lymphocytes and perhaps granulocytes and monocytes. Preliminary characterization of this receptor has shown it to migrate as a single 50,000-dalton band on SDS-PAGE analysis under reducing conditions (120). Binding of factor H to lymphocytes via this receptor is said to trigger factor I release from the cells, and exposure of monocytes to factor H increased NBT reduction and chemiluminescence. Lymphocytes, PMNs, and platelets also possess a receptor for C1q (121). There may be as many as 10^6 C1q re-

ceptors per neutrophil. The physiologic significance of this receptor is also unknown, but it is tempting to speculate that it may have a role in adherence of classical pathway activators to phagocytic cells. Since the C1 inhibitor binds to C1r and C1s and causes their release from macromolecular C1, C1q may remain attached to the classical pathway activator and be exposed to cellular receptors. Interaction of C1q with its receptor on monocytes and macrophages has also been shown to activate the cell to facilitate phagocytosis of both IgG-coated ligands and C3b/C4b-coated ligands. A macrophage receptor for Bb, which leads to macrophage spreading on glass or plastic surfaces, has also been reported.

Decay-Activating Factor (DAF), Homologous Restriction Factor (HRF), and Membrane Cofactor Protein (MCP)

These three membrane proteins do not act as typical receptors in that they do not promote the binding of a complement-coated target to the surface of cells expressing these molecules (although evidence is accruing that, in certain cases, cellular activation signals may be delivered by ligation of these glycoproteins). Nevertheless, these molecules do interact specifically with complement activation products and they play an essential role in preserving cellular integrity, preventing lysis of innocent bystander host cells resulting from spontaneous or induced complement activation.

Decay-accelerating factor (DAF) is a 70-kd membrane protein that is linked to the cell membrane by the diacylglycerol moiety of a phosphatidylinositol molecule which is covalently attached via a glycosidic linkage to the carboxy terminus of the protein (122). DAF inhibits formation of the classical pathway C4b2a convertase by interacting with C4b and preventing C2 binding, and also by destabilizing the convertase once it forms and enhancing its rate of decay. It also inhibits formation and promotes decay of the alternative pathway convertase, although it is somewhat less effective in this regard. DAF does not act as cofactor for cleavage of C3b or C4b (123). The molecule is present in the membrane of all blood cells and endothelial cells and exists in two forms, which differ in size and which are thought to result from alternative splicing of the terminal portion of the gene encoding DAF (124). One form of the protein is processed to provide the phosphoinositide membrane linkage and the other is truncated and released from the cell without the membrane anchor moiety. It is also reported that the form of this protein found in neutrophils is slightly heavier than that found in erythrocytes. Like HRF, this phospholipid-anchored membrane-protective molecule is missing from the cells in patients with the disease paroxysmal nocturnal hemoglobinuria (PNH) (125,126). These abnormalities appear to account for at least a portion of the exquisite complement sensitivity of erythrocytes in PNH and are likely responsible for chronic intravascular hemolysis in this disease.

Homologous restriction factor is a 65-kd regulatory pro-

tein which is also phospholipid anchored and has been studied thus far on lymphocytes and erythrocytes, although it has been shown to be present on monocytes, neutrophils, and platelets as well (127,128). It interacts with both C8 and C9 of the membrane attack complex to prevent successful insertion of the complex through the membrane bilayer, thus protecting cells from the late-acting steps in complement attack. It derives its unusual name from the observation that, in the case of each species thus far studied, the factor recognizes and interacts with homologous C8 and C9 far better than heterologous C8 and C9. The presence of this factor in cell membranes would appear to explain the fact that complement proteins are far more effective at lysing cells of heterologous species than they are at lysing cells of the homologous species. HRF is also reported to inhibit the action of the cellular cytotoxin termed perforin or cytolysin, a molecule found in large granular lymphocytes which has significant homology to C9 and which has been reported to cause cell lysis (129).

Yet another membrane protein termed membrane cofactor protein (MCP) or gp45-70 has been reported in the membranes of most blood cell types, but not erythrocytes. In addition, it is reported to be present on epithelial cells, fibroblasts, and endothelial cells. This protein binds to C3b and iC3b but does not appear to have sufficient affinity to act as a complement receptor per se. It does, however, facilitate the factor 1-dependent degradation of C3b to iC3b and may in fact be more efficient in this regard than any of the previously identified fluid-phase or membrane cofactors (130). Interestingly, it does not have decay-accelerating activity for either the C3 or C5 convertases.

MOLECULAR BIOLOGY, SYNTHESIS, AND DEFICIENCIES OF COMPLEMENT PROTEINS

Within the past decade the molecular biology of the complement system has come under intense scrutiny. DNA encoding nearly every component of the cascade, as well as regulatory factors and cellular receptors, has been cloned and sequenced. Chromosomal mapping of the complement genes and studies of the regulation of synthesis of several components have been performed with molecular probes. An encyclopedic review of these data is beyond the scope of this chapter and is available elsewhere (131). We treat several major themes which have emerged from these studies.

The C3b/C4b Binding Protein Superfamily

A large number of plasma and membrane proteins interact with C3b or C4b. These proteins are important in complement activation, regulation of the cascade, or as cellular receptors. They include factor H, C4BP, CR1, CR2, DAF, gp45-70 (MCP), C2, and factor B (132,133). A close relationship between these proteins was first suggested for the regulatory plasma proteins H and C4BP,

and the regulatory and membrane receptor protein CR1, on the basis of classical genetic techniques. By studies of electrophoretic polymorphic variants, the genes for H, CR1, and C4BP were found to be very closely linked in the human. As molecular probes and eventually full-length cDNA clones for these proteins have become available, a gene superfamily has emerged. At least the CR1, CR2, C4BP, H, and DAF genes are located on the long arm of chromosome one in the human (134). More importantly, each member of this group has been shown to contain from 8 to over 30 short homologous repeating units approximately 60 amino acids in length. These repeating units generally appear in sequence and occupy the amino terminal portion of the protein (133). There are eight or nine highly characteristic conserved amino acids, including four cysteines, as well as conserved hydrophobic regions. The deduced structure of CR1 has shown its 28 to 33 short consensus repeats to be organized into longer homologous repeats, each of which contains seven of the shorter segments. Duplication en bloc of one of these longer homologous repeating segments is thought to have given rise to at least one of the more common electrophoretic variants (103,131). The deduced structure of CR2 appears similar. The CR1, CR2, C4BP, and DAF genes are very closely spaced and share a higher degree of homology, while factor H is not as closely linked and is also more dissimilar in primary sequence (134). The C3b/C4b binding capacity and cofactor activity of these proteins have been explicitly shown to reside in the repeating units in the case of H and C4BP, and this relationship is presumed to hold true for the remainder of the family. While less well studied, the organization and structure of the murine C3b/C4b binding protein genes appear similar.

C2 and factor B, which also bind and interact with C4b or C3b, each contain three of the typical short consensus repeats at their amino termini (133,135). The carboxy terminal ends of these proteins confer serine protease activity. C2 and factor B are encoded on chromosome 6 in the human, within the major histocompatibility complex (MHC), and are discussed further later. Two other complement components, C1r and C1s, have also been found to have two of the homologous repeating units at the carboxy terminal end of their noncatalytic heavy chains. Several noncomplement proteins: β_2 -glycoprotein 1, factor XIII, and the (p55 chain of) IL-2 receptor also contain

several copies of the short homologous repeating unit. Careful studies of genomic clones for several proteins in this superfamily indicate that each short repeat may be encoded by a discrete exon. The functional significance of these structures and the factors underlying the presumptive multiple gene duplications responsible for this family remain to be determined.

The MHC-Linked Complement Genes

The genes encoding C2, factor B, and C4 lie in a 120-kb stretch of DNA between the HLA-DR and HLA-B loci on chromosome 6 in the human (131) (Fig. 5). The C2 and factor B genes are less than 1 kb apart. These two genes demonstrate high primary sequence homology and each encodes several amino terminal short homologous repeats linked to a carboxy terminal serine protease domain as described previously. The exon structure of the DNA encoding the protease domain of factor B and C2 is similar, but not identical, to that of other serine proteases. The C2 and factor B genes contain a unique exon encoding a region of polypeptide chain between the Asp and Ser residues of the active site that is not found in other serine proteases and share an overall 33% homology in their enzymatic domains. Thus C2 and factor B almost certainly represent a gene duplication (135).

Approximately 30 kb distant from the C2 and factor B genes lie two separate C4 loci, each associated with a gene encoding the noncomplement protein steroid 21-hydroxylase. The two C4 genes encode the two isotopic variants C4A and C4B. Fragments of C4A and C4B deposited on red cells are responsible for the HLA-linked Rodgers and Chido blood group antigens, respectively. The homology between C4A and C4B is approximately 99%, with perhaps as few as six amino acid substitutions defining the two isotypes. These isotypic variations are clustered in the portion of the C4 molecule which gives rise to the C4d fragment and contains the reactive thioester site (136). The functional consequence of the substitutions appears to be a predilection of C4A to form amide bonds with proteins, whereas C4B preferentially forms ester bonds with carbohydrate moieties. In studies of erythrocyte lysis, C4B is far more active than C4A. Multiple allotypic variants of each C4 isotype exist, so that a normal com-

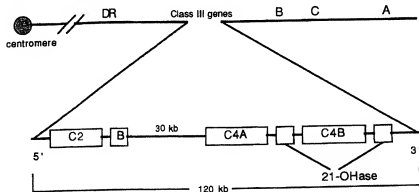


FIG. 5. A map of the MHC-linked complement genes (class III MHC gene products) on the short arm of chromosome 6 in the human. The arrangement of the HLA, A, B, C, and DR loci is shown relative to the complement genes. The expanded 120-kb segment shows the relative position and size of the C2, factor B, C4A and B, and 21-hydroxylase (21-OHase) genes. Not drawn strictly to scale.

plement of C4 genes may encode up to four distinct forms of the protein. Furthermore, null alleles at both loci are fairly common, and 10 to 15% of the normal population may carry at least one null (or Q0) C4 allele. About half of null alleles thus far examined result from large deletions which involve most or all of one C4 gene and an adjacent 21-hydroxylase gene. While less well documented, evidence is also accruing that a significant number of haplotypes in the general population may contain three C4 loci. The high frequency of both large deletions and apparent duplications has led to the proposal that unequal crossover events between the C4 loci of sister chromatids during meiosis may be responsible for such haplotypes with one or three C4 genes (137). In the mouse, only one functional C4 product is found. A second C4-like protein is encoded in the major histocompatibility complex, and expression of this protein is under the control of sex hormones (sex-limited protein or SLP). Absence of C4 function in SLP is probably related to accumulated sequence changes near the C1s cleavage site (131).

Because of their proximity in the genome, specific combinations of C2, factor B, and C4 allelic variants tend to occur together. Crossovers within these combinations are uncommon, and they tend to be inherited as units referred to as complotypes (131). Complotypes can serve as markers for even larger units which are called "extended haplotypes" and include the HLA A, B, C, and DR loci. These are usually inherited en bloc and occur in the general population at frequencies higher than those predicted by their physical proximity alone. It has been suggested that crossovers within extended haplotypes are actively suppressed, but the mechanisms and selective pressures responsible for this remain speculative.

Biosynthesis of Complement Components

Over the last several decades there has been a steady accumulation of data relating to the biosynthesis of complement components. In the human, the liver appears to play a preeminent role in the synthesis of most of the components. This has been shown clearly for C3, C6, C8, and factor B by documenting a switch in the circulating allotypes of these proteins to those characteristic of the donor after orthotopic liver transplant (138). Notably, the recipient's complement component allotypes do not disappear completely after such surgery, suggesting extrahaptic synthesis as well. Data regarding the site of synthesis of most other components are derived primarily from *in vitro* studies of primary tissue cultures or established cell lines, both animal and human. On the basis of such information, it appears that functional C1 macromolecules are produced by a variety of epithelial cells of endodermal origin, and these cells, as well as macrophages, are thought to be an important locus of *in vivo* production (139). The source of virtually all the other components studied appear to be the liver or the mononuclear phagocyte system or both (140). While mononuclear phagocytes probably contribute little to total plasma complement levels, their output may be of considerable importance at local sites of inflammation.

Regulation of complement protein biosynthesis is complex. The majority of studies have been carried out with primary macrophage cultures and hepatocyte or macrophage-like cell lines. Synthesis is responsive to a wide variety of modulators including immune complexes, ambient complement protein levels, cytokines, neuropeptides, histamine, and arachidonate metabolites, and the responses evoked are both protein and tissue specific (140). Regulatory mechanisms have been demonstrated at the pretranslational, translational, and post-translational levels in various *in vitro* systems. At the clinical level, the majority of complement proteins are elevated in plasma during acute-phase responses. Careful studies of regulation of C2 and factor B synthesis using molecular probes are in progress. Despite divergent, independent responses of the synthesis of these two proteins to a variety of stimuli, it appears that at least one segment of DNA 3' to the C2 coding sequence is essential for expression of both genes (141). It is interesting to note that at least two complement proteins, C1 and C8, are composed of two or more gene products which are assembled non-covalently in the extracellular compartment after secretion. The plasma pool of these components exists as an equilibrium mixture of complexed and disassembled subcomponents, and synthesis of the various subcomponents is clearly separable in both *in vitro* systems and genetically deficient individuals.

Complement Deficiency States

Complement deficiency states are distinctly uncommon, and this low frequency points to the strong selective pressure favoring maintenance of an effective complement cascade. Complement protein alleles, including null alleles, show codominant behavior. Thus heterozygotes for null alleles have roughly half-normal levels of the components in question and are usually clinically normal (with one exception—see later discussion). The consequences of homozygous deficiency states divide roughly in accordance with the portion of the cascade involved. Humans with deficiencies of the alternative pathway are highly susceptible to a variety of pyogenic bacterial infections (presumably due to failure of C3-dependent opsonization), whereas those with terminal component defects have a poorly understood isolated propensity to disseminated neisserial infections. A substantial proportion of individuals with terminal component deficiencies, especially C9, are, however, entirely well. Autoimmune disease, presenting as glomerulonephritis or systemic lupus erythematosus-like syndromes, is seen in deficiencies affecting all three portions of the complement cascade but is the particular hallmark of classical pathway defects (142,143). Such disorders are seen in over half of individuals with C2 or C4 deficiency. The genesis of this striking relationship is unclear. It has been proposed that C2 and C4 deficiencies profoundly alter the disposition of immune complexes and thereby promote both end-organ pathology and persistent immunoregulatory abnormalities (144). It is notable, however, that heterozygous family

members of C2-deficient patients and humans whose genotype includes even one null allele (especially C4AQ0) among the four C4 genes have increased incidences of autoimmunity (142). This finding, and the relationship of C2 and C4 genes to the MHC loci, has raised the question of additional disease-susceptibility genes linked to the null complement alleles. These questions have not been resolved.

Deficiency of C1 inhibitor in the human is clinically manifest in heterozygotes as the syndrome of hereditary angioedema (145). Affected individuals are subject to recurrent localized soft-tissue swelling. C1 inhibitor is an important regulator of the clotting, kinin-generating, and fibrinolytic enzyme systems in addition to the complement cascade. Chronic consumption of the inhibitor by these enzyme systems outstrips the output directed by one normal gene, and C1-inhibitor titers fall well below half-normal levels. Failure of homeostatic regulation of one or more of the above protease systems is then believed to generate a vasoactive mediator(s) which results in angioedema. The precise mediator has been extensively sought but remains uncertain.

A number of animal models of complement deficiency are available. These include C2-, C3-, and C4-deficient guinea pigs, C3-deficient dogs, C5-deficient mice, and C6-deficient rabbits (146). These have been useful in elucidating the role of complement in opsonization and in confirming abnormalities of the immune response to prototypic antigens. Some of these animals exhibit clinical diseases or subclinical serologic abnormalities that mimic their human counterparts.

CONCLUSION

As recently as 1969, Macfarlane Burnet wrote in his book *Cellular Immunology*:

Since the studies of Bordet, Ehrlich and Wasserman, the concept of complement as an essential part of the mechanism of immunity has progressively been replaced by a rather uncertain decision that the classic phenomenon of complement lysis of red cells is a laboratory artifact of no real significance for immunity.

We hope that it is clear that our understanding of complement has come a long way since that time. We are now certain that the complement proteins play a critical role in host defense and the development of autoimmunity. We believe it likely that these proteins will prove to be important in control of a number of steps in the immune response as well. The fact that very few individuals are missing any of the many proteins and the fact that the proteins show remarkable evolutionary stability suggest that their further study will reveal new important control functions. Now that the chain structure of most of the proteins and cleavage fragments is known and the amino acid sequence of many of the proteins is established, understanding their interactions and the biologic consequences of their activation will provide one of the major challenges of the next decade.

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27

Functions and Mechanisms of Lysis Induced by Cytotoxic T Lymphocytes and Natural Killer Cells

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It is quite fascinating that while metabolically and structurally different cells can live together harmoniously, certain specialized cells are also capable of damaging and even killing neighboring cells. Killing of one cell type by another through contact interaction constitutes a major effector arm of self-defense of the immune system. The

major immunologically relevant cytocidal cells other than macrophages are cytolytic T lymphocytes (CTLs), natural killer (NK) cells, and lymphokine-activated killer (LAK) cells. This chapter deals with cellular and molecular mechanisms involved in this wondrous mechanism of natural immunity, which assiduously protects the body

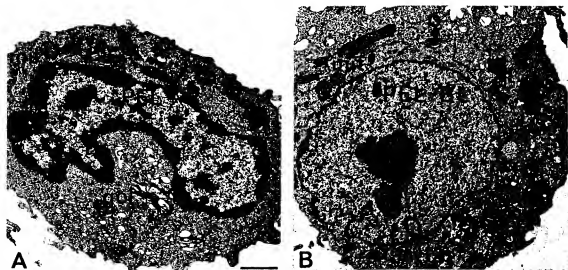


FIG. 1. Electron micrographs of nongranulated and granulated CTLs. The nongranulated CTL (A) are BALB/c anti-EL4 alloreactive peritoneal exudate cytolytic lymphocytes (PELs) (Berke et al., ref. 17). The PEL-blast (PEL-BL) (B) is derived from PELs cultured in the presence of interleukin 2 (IL-2) (Berke and Rosen, ref. 88). Note the presence of osmiophilic granules (gr) in the PEL-blast and their absence in the PELs. gol, Golgi apparatus; mit, mitochondria. Bar represents 621 nm for A and 1960 nm for B. Electron microscopy by D. Rosen.

against viral, certain bacterial, and perhaps even cancerous diseases. On the negative side, however, killer lymphocytes form the primary obstacle to be overcome before tissue and organ transplants can be spared from rejection and before tissue damage in autoimmune diseases can be prevented.

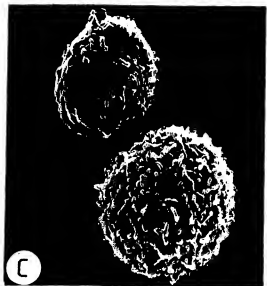
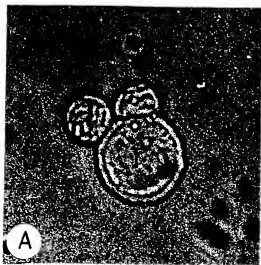
A major part of this chapter is devoted to the mechanism(s) whereby lymphocytes recognize and kill other cells, the subject of many previous investigations (reviews in refs. 1–4) since Govaert's original observation of cytotoxic lymphocytes in kidney allograft rejection. This topic is preceded by an introduction to the generation and classification of cytotoxic cells (reviews in refs. 5–8) and is followed by an assessment of their activities. We also discuss the function of cytotoxic cells in graft rejection, virus immunity, cancer immunotherapy, and the induction of tissue damage in autoimmunity (reviews in refs. 9–11).

CYTOTOXIC CELLS: THEIR GENERATION, RECEPTORS, AND MARKERS

Although predicted earlier, the discovery of specifically reactive cytotoxic lymphocytes must be credited to Govaert (12). The demonstration that sensitized cytotoxic

lymphocytes express the Thy-1 (formerly termed θ) antigen (Ag) on their cell surface resulted in their classification as cytotoxic T lymphocytes (CTLs) (13). Subsets of T lymphocytes were also discovered later. It is now known that the CD4 and CD8 cell surface molecules (L3T4 and Lyt2, respectively, in the mouse) are expressed on two mutually exclusive subsets of mature T lymphocytes. Most T helper cells (T_H) express CD4, whereas most cytotoxic/suppressor T cells (T_{cy}) express CD8. This correlation, however, appears not to be strict, as cytotoxic CD4⁺ T lymphocytes have been described (14), and cytotoxic CD4⁺ effectors may play a role in down regulating immune responses by killing autologous, antigen-presenting cells (B cells, macrophages). CD4⁺ cells always recognize antigen plus class II MHC molecules (even when they kill) and CD8⁺ cells recognize antigen plus class I (even when they do not kill). Some CTLs and most NK cells appear as large granular lymphocytes (LGLs) while mature *in vivo* primed CTLs are small to medium sized (8 to 12 μ m), nongranular lymphocytes. Transformation from the latter to the former type (Fig. 1) can be induced by interleukin 2 (IL-2) and probably by IL-4. Three distinct types of cytotoxic lymphocytes have been defined (15). The first are Ag-specific CTLs restricted by class I major histocompatibility complex (MHC) molecules that recognize the target through an idiotypic T cell receptor (Ti) associated with another cell

FIG. 2. CTL–target cell conjugates. Small cells are BALB/c anti-EL4 peritoneal exudate CTLs, while the larger ones are EL4 target cells (T) of C57BL/6 origin (Berke et al., ref. 53). A and B: Light microscopy. C and D: Scanning electron microscopy. E and F: Transmission electron microscopy showing contact region and interdigitations. Arrows in D point to interdigitations.



surface multimolecular complex (CD3) (16). These MHC-restricted CTLs can be defined by the rearrangement of their T cell receptor (TcR) α and β genes, the expression of the CD3-Ti molecular complex on the cell surface, and their cytolytic function, which is both Ag specific and MHC restricted. The second type consists of broadly specific CTLs that recognize their targets without MHC restriction, but nonetheless via the CD3-Ti complex. The third type consists of non-MHC-restricted NK cells that recognize and kill certain target cells (NK sensitive) via an as yet undefined receptor. Common to all three forms of lymphocytotoxicity is an initial lymphocyte-target cell adhesion step (conjugation formation) (see Fig. 2), ultimately leading to target lysis. The assessment and mechanism of target cell recognition and lysis is described in a later section of this chapter.

These three distinct effector cell types may also engage in alternative target recognition pathways mediated by exogenously added factors such as antibodies or lectins. For example, in antibody-dependent cellular cytotoxicity (ADCC), the effector lymphocytes express Fc receptors, and target cell recognition is mediated by the interaction of these receptors with the Fc portion of antibody bound to the target cell. Cells capable of performing ADCC have previously been named K cells, but it is now clear that both NK cells and CTLs expressing Fc receptors for IgG (Fc γ R) as well as macrophages and monocytes can mediate ADCC. Non-MHC-restricted, non-specific cytotoxicity can also be induced by CTLs, if the effector and target cells are allowed to react in the presence of antibodies to one or more components of the Ti-CD3 complex or the mitogenic plant lectins concanavalin A (Con A) or phytohemagglutinin (PHA), which provide both effective intercellular bonding and enable triggering of the effector cell lytic machinery.

Cytolytic T Lymphocytes (CTLs)

Effector CTLs are generated in response to allogeneic cell surface MHC determinants (as in allotransplantation), mitogenic lectins, chemically modified or virally infected autologous or syngeneic cells, and tumor-associated Ag. The generation of CTLs from their precursors involves a complex series of events and signals—not all of which are fully understood—ultimately resulting in the production of effectors, capable of specifically recognizing and lysing the target (7,8) (Fig. 3). Schematically, it is believed that resting CD8⁺ CTL precursors (Lyt2 in the mouse) are triggered directly by either foreign class I MHC surface molecules (in allogeneic responses) or by nominal Ag (viral, bacterial) in conjunction with class I MHC molecules (in MHC-restricted responses) (first signal). Within several hours after onset of activation, surface expression of interleukin 2 (IL-2) receptors and blast transformation occur prior to cell division. Proliferation of these activated CTL precursors will not occur unless a second signal, IL-2, is provided. To this end, precursors of T_H cells (CD4⁺; L3T4⁺ in the mouse) are triggered by exposure to allogeneic class II MHC molecules alone (in allogeneic

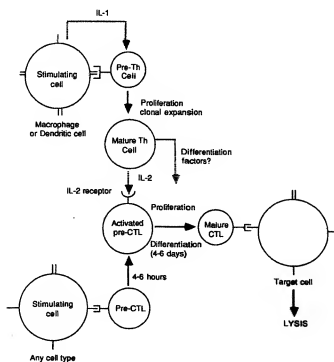


FIG. 3. Cell interactions in the generation of cytotoxic T lymphocytes. Pre-CTLs (lower part of the figure) need at least two signals to become functionally mature. Signal 1 is provided by the interaction of the pre-CTLs' antigen receptor with a MHC class I Ag on an allogeneic stimulating cell (or with foreign Ag presented in conjunction with self-MHC class I Ag in MHC-restricted responses). As a result of this signal, the pre-CTLs become "activated" and express receptors for IL-2. However, the activated CTLs will not divide unless they are provided with a source of IL-2 (second signal). This is normally supplied by a nearby T helper (T_H) cell (upper part of the figure), activated to produce IL-2 by interaction with class II-bearing, IL-1-producing allogeneic stimulating cells, most likely a dendritic cell or macrophage, or with foreign Ag presented in conjunction with MHC class II Ag in MHC-restricted responses. When the activated pre-CTL encounters IL-2, it divides and matures into a fully cytotoxic cell, possibly under the influence of additional (differentiating) factors provided by the T_H cell. The mature CTLs can then attack and destroy cells bearing the same class I antigens as the original stimulating cell. I and II refer to class I and class II MHC antigens, respectively. (Adapted from Wagner et al., ref. 8, and Bach et al., ref. 25.)

responses) or to complexes of class II molecules or nominal antigens (as in virus infection) on dendritic cells or macrophages. Interleukin 1 (IL-1) secreted by the macrophages is presumed to activate the T_H to produce IL-2, which in turn induces proliferation of the activated pre-CTLs (second signal). Under the influence of IL-2 *in vitro*, activated, proliferating lymphoblasts acquire azurophilic granules and serine protease activity. These activated CTLs are cytotoxic and they gradually differentiate into small to medium sized effectors that express potent, spe-

cific cytolytic activity but no cytolytic granules, and finally into memory CTLs. In secondary (anamnestic) CTL responses, the participation of T_H (and IL-2 production) does not appear to be essential for effector (memory) cell activation.

Allospecific CTLs: *In Vivo*

In experimental animals, alloreactive CTLs are generated in response to transplanted allogeneic normal tissues or to transplantable allogeneic tumors (frequently, mouse leukemias are used). One convenient system utilizes the intraperitoneal injection of allogeneic tumor cells where primary rejection of the tumor cells by CTLs can be studied in detail. While spleen or lymph node cells derived prior to, during, or shortly after this allogeneic rejection provide a good CTL source (13), peritoneal exudate cells collected shortly after primary or secondary rejection of an intraperitoneal tumor allograft are an excellent source of highly potent, specific CTLs (17,18). Such cells are capable of binding to and lysing target cells *in vitro*, as determined by the conjugation (target binding) and the ^{51}Cr release (target lysing) assays, respectively (17,18) (Fig. 4).

That MHC class I differences *alone* are required and sufficient for allogeneic CTL generation has been dem-

onstrated by the subtle mutational event(s) of H(Z1) mice—later called bml—where two amino acid substitutions in MHC class I products (class II molecules of the mutant and wild type are identical) give rise to CTL production, cellular alloreactivity, as demonstrated by skin and tumor allograft rejection, and mixed-leukocyte reaction (MLR) (19–21). Interestingly, the cellular alloreactivity and generation of CTLs directed against the mutated MHC class I molecules of bml mutant mice occurs in the absence of detectable alloantibody production (21). Because of distinct cross-reactivities of mutant-specific CTL, it appears that the MHC class I element(s) detected by CTLs in bml mutants is conformational and not sequential (22).

Allospecific CTLs: *In Vitro*

Ginsburg and colleagues were first to generate specific cytotoxic lymphocytes *in vitro* (23). They showed that cocultivation of unprimed lymphocytes with stimulating fibroblast monolayers of various mouse and rat strains (xenogeneic and later allogeneic combination) resulted in lymphocyte transformation and production of strain-specific cytotoxic lymphoblasts capable of lysing ^{51}Cr -labeled fibroblast monolayers antigenically identical to the original stimulating monolayers (23,24). Subsequently, it was shown that mixed leukocyte cultures (MLCs) of allogeneic spleen, lymph node, or peripheral blood cells, which have been incubated *in vitro* for a few days, provide an excellent system for studying alloreactivity and CTL production *in vitro* (5,25). Precision was given to this response when it was found that prior treatment of the stimulator cells by mitomycin C or X-irradiation results in one-way MLCs, not unlike MLCs between lymphocytes of homozygous parental and F1 hybrid cells (25). Adoptive transfer of immunity *in vivo* by cytotoxic cells sensitized *in vitro* has been demonstrated (5,26), showing the effectiveness of the *in vitro* primed cells in an *in vivo* setting. CTL generation in MLCs has therefore been used as a well-characterized model system in which both the afferent and efferent phases of T-cell-mediated immunity can be studied. Anamnestic (memory) CTL responses can also be induced and studied *in vitro* by reexposure of resting MLC cells to the original stimulating cell (5), and even to polyclonal stimulators such as mitogenic lectins which result in cell proliferation and prompt reappearance of high levels of specific CTL activity. Moreover, cytotoxic reactivity of these anamnestic CTLs can be induced even when DNA synthesis is completely blocked, for example, by cytosine arabinoside (27).

MHC-Restricted CTLs

Cytotoxic cells specific for a nominal (non-MHC) Ag presented in conjunction with a particular MHC (class I or II) Ag are termed MHC restricted (28–30). Specific MHC-restricted CTLs directed against combinations of class I MHC molecules and small chemical groups [e.g.,

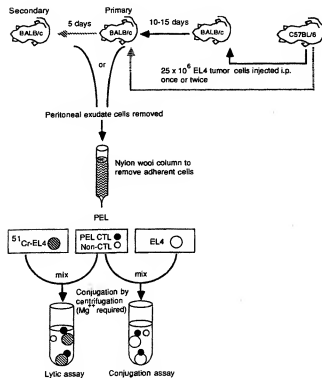


FIG. 4. Generation and testing of the lytic and conjugating activity of *in vivo* primed peritoneal exudate CTLs. (Adapted from Berke, ref. 54, and Berke and Amos, ref. 57.)

dinitrophenyl (DNP), fluorescein isothiocyanate (FITC)] are obtained following *in vitro* cultivation of lymphocytes with autologous lymphocytes modified by the particular chemical (29). MHC-restricted, tumor or virus specific CTLs can be obtained from the lymph node or spleen cells of virus- or tumor-injected animals. The *in vitro* restimulation of these cells with tumor cells or virus-infected cells (28) results in an enhanced CTL response. MHC-restricted CTLs to minor histocompatibility Ags, β_2 -microglobulin, and to other cell surface determinants have also been described (31).

Polyclonal CTLs

Polyclonally activated CTLs can be generated following Con A- or PHA-induced stimulation of lymphocytes. Other polyclonal stimulators include Staphylococcal enterotoxins (e.g., SEB), antibodies to CD3 (a T-cell receptor-associated multimolecular complex), as well as other T cell mitogens. Within 24 hr of such stimulation, small lymphocytes undergo blast transformation and commence cell division. The resultant blasts exhibit a low level of cytotoxic activity against a wide range of target cells (32); this cytotoxicity is enhanced in the presence of the lectin. The low cytotoxicity is because most if not all clones are stimulated; namely, any one clone is represented at a low frequency. Interestingly, *in vitro* stimulation by these mitogenic lectins of memory spleen cells derived from previously alloimmunized mice results in an Ag-like anamnestic response and production of MHC specific CTLs (33,34), probably due to the selective advantage of memory CTLs in response to mitogenic stimulation. Thus lectins can induce both specific and seemingly nonspecific CTLs, the ratios of which depend on previous antigenic stimulation of the responding cells and the system employed.

IL-2-Dependent CTL Lines and CTL Hybridomas

Today, cloned, IL-2-dependent CTLs and NK cell lines (35) are important sources of effector cells for studying (a) the phenotypic expression of differentiation Ag, (b) fine Ag specificity of T cell responses, (c) effector cell activation and cytotoxic activity, and (d) the structure and molecular biology of T cell receptors. One drawback of CTLs and NK clones maintained *in vitro* in IL-2 is that these cultured effectors may develop unexpected specificities (36). For example, cloned CTLs can evolve into cells with NK-like granules and NK-like target specificity (37) but which are not NK cells because they continue to express Ti-CD3 determinants (15).

With the advent of "immortal" antibody-producing B cell hybridomas, the generation of CTL hybridomas became an obvious "next step." Early attempts to somatically hybridize CTLs and tumor cells, thereby forming hybridomas, were unsuccessful. The failure was attributed to polyethylene glycol-mediated CTL lysis of the fusion partner. Nevertheless, successful production of CTL

hybridomas was reported by two groups working simultaneously but independently (38,39). The CTL hybridomas generated by Kaufmann et al. (39) grow without externally supplied IL-2, exhibit specific lytic activity, and express the Thy-1 marker and the T cell receptor (Ti) α and β chains. Although expressing innate lytic activity, the cytotoxic capacity of and IL-2 production by these CTL hybridomas is augmented significantly upon *in vitro* stimulation by mitogenic lectins (e.g., Con A) or specific antigenic cells (40,41), suggesting that the hybridomas are derived from and represent memory CTLs.

Natural Killer (NK) Cells and Lymphokine-Activated Killer (LAK) Cells

These cells, which exhibit LGL morphology, probably play a role in tumor resistance, host immunity to viral and perhaps other microbial infections, and in the regulation of lymphoid and other hemopoietic cell populations (42). NK cells do not exhibit rearrangement of the genes that code for the β chain of the TcR and do not express cell surface CD3 determinants. However, they usually do express CD16 and Leu19 (NKH-1) antigens and Fc receptors. The cell lineage of NK cells is uncertain. The target cell specificity of NK cells suggests that the cytolytic activity of a NK population is not due to a single cell type but is rather the summation of the lytic activities of several different cell lineages at particular stages of maturation and activation. A consistent feature of NK cells which has enabled their isolation, examination, and comparison with other cell types is their association with a subpopulation of cells, the LGLs (reviewed in ref. 42). LGLs are present in the peripheral blood, spleen, and liver of unprimed animals, including athymic "nude" mice and humans. By centrifugation on discontinuous Percoll gradients and elimination of cells that form high-affinity rosettes with sheep red blood cells, a population consisting of 95% LGLs can be obtained from peripheral blood. It should be noted that not all NK cells may be LGLs, nor do all LGLs exhibit NK activity. Certain transplantable tumor cells [e.g., rat NK (RNK) leukemia] exhibit considerable NK activity and cytoplasmic granulation (42,43). Some target cells are highly susceptible to NK-induced lysis (e.g., YAC in the mouse and K562 in humans) while others (e.g., EL4 and Daudi in humans) are refractory. The molecular basis for this differential susceptibility is not well understood. The recent conversion of NK-resistant tumor cells into NK-sensitive targets upon fusion with liposomes containing NK-sensitive membrane determinants (44), or during B cell differentiation (45), may help define the molecular nature of the target cell determinants recognized by NK cells.

Incubation of peripheral blood lymphocytes with IL-2 results in the production of lymphokine-activated killer (LAK) cells, initially thought to be a unique cell population capable of lysing fresh tumor cells but not normal or NK-sensitive target cells (46). Recent evidence suggests that LAK activity can be attributed primarily to IL-2-activated NK cells (47). A separate class of natural cytotoxic (NC) cells, in addition to NK cells, have been

demonstrated in mice. NC cells differ from NK cells with regard to their cell surface characteristics, target selectivity, and organ and strain distribution. In view of the probable involvement of tumor necrosis factor (TNF- α) in lysis induced by NC cells, they may be regarded as effectors of the myelomonocytic (monocytes) series, although highly purified NK cells can also produce TNF (48).

ALLOGENEIC RESPONSES, CLONAL SELECTION, AND MATURATION OF CTLs

The specificity and molecular nature of the CTL Ti receptor are comparable to that of membrane-bound Ig receptors of B cells, although a more complex series of molecular interactions appears to be involved in T cell triggering (16). The application of Burnet's clonal selection and expansion theory to CTLs is complicated by the finding that unprimed animals already contain large numbers of committed T cells reactive to a given alloantigen in mixed lymphocyte reactions. At least one and according to some estimates up to 5% of all T cells from unprimed animals respond to a given alloantigenic challenge. However, a given alloantigenic difference may entail a multitude of antigenic epitopes. Some studies even support the extreme view that no clonal expansion (cell division) is required in an *in vivo* allogeneic T cell response, suggesting that clonal activation alone without expansion may be required and sufficient. However, direct assessment of effector cell replication during CTL production *in vivo* (49) has supported the concept of clonal expansion in the usual type of CTL responses as observed *in vivo*. In that system, the proliferative response of peritoneal exudate CTLs of mice responding to an allogeneic tumor injected intraperitoneally was monitored by administering ^3H -thymidine during induction of the CTL response *in vivo*. Almost all the specific conjugate-forming peritoneal CTLs obtained in this case were ^3H labeled on autoradiography and thus clearly were the products of dividing cells. Thus the clonal selection and expansion model is applicable to at least one primary *in vivo* alloimmune T cell response resulting in CTL production. However, one must remember that the allogeneic CTL response is biologically artificial; it seems much more likely that MHC-restricted, "self + X" CTL responses *in vivo* would reflect true clonal restriction and expansion à la Burnet.

The observed cytolytic capacity of various lymphoid cell populations, as a function of time after immunization (50–52), is also pertinent to the issue of clonal T cell activation and/or selection. Changes in the lytic potential of a given lymphoid population may be due to alterations in numbers of effector CTLs and/or in their individual cytotoxic activity. A correlation between the number of cells capable of binding target cells and the population's lytic capacity has been demonstrated (Fig. 5). The frequency of peritoneal exudate CTLs capable of binding to and lysing target cells increases from a background of 2–5%, up to 35% within 11 days of primary intraperitoneal alloimmunization and then decreases (53). These findings sup-

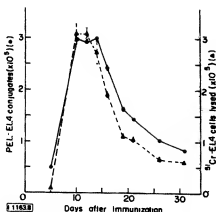


FIG. 5. Conjugation and cytotoxicity as a function of time after a primary alloimmunization. BALB/c mice were inoculated with EL4 intraperitoneally. PELs were isolated at the indicated times and assayed for their conjugation (●) and lytic activity (Δ). (From Berke et al., ref. 53 with permission.)

port the view (54) that alterations in cytolytic activity following alloimmunization are due to changes in frequency of effector CTLs capable of binding to and lysing the target, rather than to obvious variations in the lytic capacity of individual cells, that is, that effector CTL in an immunized population are either cytotoxic or not (i.e., cytotoxicity is "quantal").

CTL maturation during primary and anamnestic allogeneic responses can be evaluated by examining the specificity of target cell binding by CTLs, the cytolytic and recycling ability of individual CTLs, and the avidity of effector–target cell conjugation (50). The target MHC haplotype and subloci specificity of responding CTLs are preserved after repeated immunizations. Likewise, the lytic rate and recycling ability of individual CTLs are not altered by repeated immunizations. However, inhibition both of CTL–target cell conjugate formation and of CTL-mediated target cell lysis by antibodies against target MHC Ags or the effector's Lyt2 determinants is less effective with tertiary CTLs, suggesting an increase in avidity of effector–target cell interaction after repeated immunization (50). A similar increase in apparent avidity is also observed during CTL priming in MLRs, as deduced from blocking by antibodies directed against CTLs and/or target determinants (27). These observations suggest that responding CTL populations are subjected to moderate selective processes upon repeated antigenic stimuli, and provide a further analogy with B cell responses in which the most avid responders are selected over time and with decreasing amounts of nominal antigen.

IN VIVO FUNCTION OF THE CYTOLYTIC LYMPHOCYTE

Graft Rejection

Early Studies

Multicellular organisms are equipped with an intricate immune system to recognize and neutralize foreign and

potentially hazardous agents, such as intracellular and extracellular parasites and neoplastic cells. Due largely to the work of Snell, Gorer, Medawar, Billingham, and Brent (reviews in refs. 13,55-57), it is now apparent that the rejection of normal (or malignant) allografts is due to a genetically determined and powerful immunological process. That allogeneic tumor fragments enclosed in diffusion chambers implanted into the peritoneal cavities of immune mice were not rejected showed the necessity for direct contact between grafted and host effector cells for rejection to occur (58). On the other hand, antibody and complement can directly lyse certain tumor targets even inside diffusion chambers (59). Mitchison's adoptive transfer experiments (60) demonstrating transference of second set allograft rejections by "primed" lymphoid cells but not by immune allosera established the cellular basis of allograft rejection. Antibody plus complement-induced tissue damage may be a vascular phenomenon, while cellular effectors are involved in response to parenchymal tissue. The "Winn assay" for measuring the antitumor activity of lymphocyte populations by injecting immune lymphocyte-tumor cell mixtures subcutaneously and monitoring tumor growth was an important step in determining the tumor-neutralizing capacity of sensitized lymphocytes in allogeneic and syngeneic ("tumor specific") systems. Govaert (12) was the first to show that canine thoracic duct lymphocytes, procured after kidney allograft rejection, had a specific, cytopathic effect on donor renal cells cultured *in vitro*. His findings were soon followed by a plethora of evidence demonstrating cytopathological effects of lymphoid cells from animals sensitized against normal or malignant tissues, including human cancer, or suffering from autoimmune diseases. *In vitro* lysis of virus-infected cells by cytotoxic lymphocytes obtained from virus-infected animals suggested their involvement in viral immunity and led to the important discovery of MHC restriction.

Allograft-Infiltrating Cytocidal Cells

Involvement of lymphoid cells in the elimination of histoincompatible cells has been established by adoptive transfer and diffusion chamber studies (55,61,62). Infiltration of the graft parenchyma by mononuclear cells, producing a destructive lesion, is a common event both in allograft rejection and in delayed-type hypersensitivity. Depending on the histological type of the transplant, its anatomical site, and the time of examination, the infiltrating cells may be of predominantly lymphoid or macrophage origin.

The cells infiltrating rabbit skin allografts and associated with the damaged graft epithelium are mainly small to medium sized lymphocytes (63). Density distribution analysis of spleen cells from mice undergoing allograft rejection reveals that large, rapidly dividing effector lymphoid cells are present in the spleen shortly after immunization, whereas small effector lymphocytes predominate at later times and during onset of rejection (64). In rodent intraperitoneal ascites allograft systems, where direct investigation of graft-associated cells is relatively

simple, the specific cytotoxic cells at the time of rejection are nonadherent, nonphagocytic, small to medium sized Lyt2^+ T lymphocytes (17,18). The role of the macrophage in this setting varies with different tumor targets from a primary effector cell to a mere scavenger. Interestingly, early (but not later) in the course of an allogeneic response, Lyt2^- negative effectors expressing nonspecific cytotoxic activity have been demonstrated (49). Although they are important topics with respect to tumor destruction, macrophage killing and vascular changes causing necrosis are outside the scope of this chapter.

Small lymphocytes enter the allograft within 1 to 2 days after transplantation and rapidly transform into large lymphoblastoid cells ("peripheral sensitization"); alternatively, sensitization occurs at a draining lymph node. Blast transformation also occurs during mixed lymphocyte reaction, an *in vitro* correlate of allograft rejection (65). That the responding lymphocytes initially undergo transformation into large blastoid cells that express lytic activity, revert to small to medium sized cells that also express lytic activity, and finally evolve into memory CTLs has been demonstrated both *in vivo* and *in vitro* (9,62). Although cytotoxic cells can be detected in lymphatic organs and within grafts shortly after allograft immunization (52,66), graft rejection is usually not apparent until 8 to 10 days after transplantation. The progression of cells into grafts can be studied by the elegant sponge procedure described by Hayry and colleagues (67). When a spongy matrix embedded with allogeneic cells is implanted into a histoincompatible recipient, it is infiltrated by host cells which can be released by squeezing the sponge, thus providing a convenient system for studying allograft infiltration. Although both T and non-T lymphocytes infiltrate the sponge, only a small fraction of the infiltrating cells are T lymphocytes, which is surprising since a potent effector T cell response is observed simultaneously in peripheral lymphoid organs, such as the spleen and lymph nodes. In heart and kidney transplants, several classes of both lymphoid and nonlymphoid cells have been reported to infiltrate the allograft. Unlike the sponge matrix, in these and similar systems, mechanical and enzymatic procedures must be employed to obtain a cell suspension from which the infiltrating cells can be obtained. Naturally these procedures may select for certain cell types, as well as damage or change the properties of the cells in question.

On the other hand, intraperitoneal ascites tumor allografts allow observation of graft-infiltrating cells without any chemical, enzymatic, or mechanical manipulations (68). In such systems, the infiltrating cells, which exhibit specific *in vitro* binding and cytolytic activity at the time of rejection, are small CTLs, although significant numbers of macrophages (up to 50% of the population) and other cell types are associated with the allograft (17). Thus it appears that both large and small lymphoid cells from allografted animals, which exhibit lytic activity *in vitro*, may be responsible for graft rejection *in vivo*.

The T Cell Subset(s) Responsible for Graft Rejection

A large body of evidence indicates that T lymphocytes are the primary mediators of graft rejection, yet the par-

ticular T cell subset involved in the actual rejection process remains controversial (9,67,69,70). The infiltration of CD8/Lyt2 type lymphocytes and the strong cytopathological component in graft rejection have implicated the direct involvement of cytotoxic T lymphocytes (CTLs). Furthermore, a good correlation between several parameters of allograft rejection (e.g., kinetics, genetics, anatomical association) and host CTL activity *in vivo* has been demonstrated. It is possible, however, that the T_h cell subset (CD4/L3T4), involved in delayed-type hypersensitivity (DTH), is at least partially responsible for some tissue damage and graft rejection. The use of antibodies to define functional T cell subsets may be misleading, since the serological division of T cell subsets may not be as restricted as once thought. In addition, some $Lyt2^+/CD8^+$ type cells have been shown to produce IL-2, a T_h characteristic, and some $Lyt2^-$ cells exhibit cytolytic capacities. Furthermore, certain cells can express more than one activity; for example, one cloned T cell line has been shown to express allo-help, cytotoxicity, and DTH capacities (71), and a cytotoxic T cell clone and a monoclonal CTL hybridoma have been shown to generate helper activity (in this case lymphokine production) (40). On the other hand, the observation that cloned $Lyt2^+$ cytotoxic cells can specifically destroy allografts *in vivo* confirms that CTLs can mediate transplant immunity in an immunologically specific, MHC-restricted fashion (72).

Testing for in Vivo Bystander Cell Lysis During Allograft Rejection

Graft rejection mechanisms mediated solely by a delayed-type hypersensitivity response, involving production of phagocyte-attracting lymphokines or tissue-destroying mediators, which act nonspecifically, rather than by specifically sensitized CTLs, are incompatible with the specificity and selectivity of the rejection episode. For example, skin grafts from allophenic mice (mosaics produced by mixing early-stage allogeneic embryos) grafted onto one parental strain resulted in rejection mainly of the melanoblasts and hair follicles that express the same H-2 as the allogeneic parent type, while those of the host H-2 type did not suffer irreversible damage (73). Furthermore, injection of allogeneic murine tumors mixed with syngeneic tumors (at a cell ratio of 500:1, respectively) resulted in selective and complete rejection of the allogeneic tumor, without affecting the syngeneic one (74). In another CTL system, lymphocyte-induced bystander lysis of cells infected with a third-party virus was also not observed. Thus CTL-mediated lysis is one of the rare cellular cytotoxic mechanisms that displays a high degree of specificity, thereby discriminating between target and bystander cells. However, it is conceivable that, under special circumstances, nonspecific in addition to Ag specific reactions initiated by CTL-target cell interactions may contribute to tissue destruction in allograft rejection, in severe forms of delayed-type hypersensitivity, and in certain viral infections.

Virus and Bacterial Infection: MHC Restriction

Virtually all cells are potential candidates for virus infection and phagocytes are the most susceptible to bacterial infections since they endocytose them. Host cells carrying viruses must be eliminated to keep virus proliferation to a minimum; however, destruction of bacteria- or virus-containing phagocytic cells may result in the dissemination of these organisms. It has been found that immunocompetent host T cells are triggered by bacterial or viral determinants only when they are presented as Ag in the proper molecular form on cell surfaces, in association with MHC class I and class II molecules. Phagocytosed bacteria are digested and their processed Ag are in the form of peptides presented by macrophages in association with MHC class II-encoded surface molecules (MHC class II restriction). These "hybrid" membrane structures induce specific T_h cell proliferation and differentiation, leading to the release of lymphokines, including macrophage-activating factor(s), which ultimately eliminate the infecting bacteria. CTL recognition of viral antigenic determinants on infected cells occurs when they appear in conjunction with MHC class I molecules on the cell surface (MHC class I restriction) (10,28,75). In general class I MHC determinants appear to present endogenously synthesized virus and antigens, while class II is involved in presenting exogenously encountered proteins (76). Anti-influenza CTLs recognize murine L fibroblasts that express viral nucleoprotein (NP) determinants, raising the question of how these originally nontransmembrane viral proteins are processed and presented so that they can be recognized by CTLs. Recent studies are consistent with the view that somatic cells bearing class I molecules are capable of degrading and presenting newly synthesized viral proteins and peptides to CTLs (77,78). The molecular nature of viral epitopes recognized by CTLs on the surface of virus-infected cells is now being resolved (77).

Virus-neutralizing antibodies are effective in preventing initial viral infections. However, cell-mediated immunity is essential to eliminate established viral infections. For example, athymic mice often suffer from persistent and progressive virus infections, probably due to their defective cell-mediated immune system. In murine lymphocytic choriomeningitis (LCM) clearance of the viral infection is effected by CTLs (79). The pathological changes induced by LCM virus have been attributed to damage of virus-infected cells by T cells since virally infected athymic mice do not show brain lesions. In influenza virus infection, whether viral spread is limited by γ -interferon released by CTLs at the site of virus infection, or solely by lysis of infected cells, is not known. However, cloned, influenza virus specific CTLs can protect mice against 10 lethal doses of the specific virus but not against one lethal dose of a non-cross-reacting virus admixed and administered with the virus against which the cloned CTLs were generated (10). These results establish the essential role of direct, specific effector cell contact with infected target cells and exclude involvement of nonspecific cytotoxic factors that work at a distance.

CTLs and NK Cells in Tumor Immunity

Repeated demonstrations of tumor specific immune responses (80-82) have formed the basis of tumor immunology and have supported the theory of "immune surveillance" against tumors. The principal assumption of this theory is that tumor specific immunocompetent host cells can be triggered by neoplastic cells to differentiate into effector cells capable of either destroying the tumor or arresting its growth. Although lymphoid cells exhibiting such effector activity have been observed in experimental animals and in some cancer patients, the precise effector cell populations involved and their specificity have not yet been determined unequivocally and their biological importance is unresolved. Furthermore, cellular immune responses against syngeneic tumors vary according to (a) the host, (b) the tumor, (c) the assays employed, (d) the inoculation site and the dose, and (e) the time and site of effector cell removal.

Analyses of effector cell phenotype, specificity, and mechanism of action against autologous tumor cells reveal that the antitumor cytotoxic response can be multiclonal, mediated by multiple mechanisms, and directed against different determinants expressed on the same tumor cells (83,84). Furthermore, the types and lytic mechanisms of effector cells involved depend on the tumor. For example, using four different syngeneic tumor systems, Haskill and co-workers (80) demonstrated nonspecific, cytostatic macrophages associated with a rat sarcoma; specific, cytotoxic, nonphagocytic, Fc-bearing lymphocytes associated with a murine mammary adenocarcinoma; cytostatic, macrophage-like cells and specific, cytotoxic lymphocytes associated with a primary murine sarcoma, and CTLs within a rapidly growing fibrosarcoma. Adherent, non-T, nonphagocytic cells capable of lysing antibody-coated chicken red blood cells have been detected in the peritoneal cavities of mice with ascites tumors. Nonadherent cytotoxic cells have been obtained from the peritoneal cavities of mice after repeated intraperitoneal injection of irradiated ascites tumors. Even a single intraperitoneal injection of ^{60}Co -irradiated leukemia cells could induce immunity to nonirradiated syngeneic tumor cells in mice (66). In this system, tumor-associated peritoneal exudate cells exhibited specific *in vitro* cytolytic activity as early as 3 days after the intraperitoneal injection of irradiated tumor, and the activity peaked on days 5 to 6.

Tumor Therapy Using LAK Cells and Tumor-Infiltrating Lymphocytes (TILs) Activated by Interleukin 2 (IL-2)

Immunizations against cancers using tumor cell vaccines and attempts to bolster immunity against cancer by immunomodulators have generally been unsuccessful. The commonly low and sometimes even total lack of cytotoxic activity of human lymphocytes against autologous tumors and the difficulty of attaining sufficient quantities

of autologous lymphoid cells whose anti-tumor activity could be boosted *in vivo* or *in vitro* have hindered the development of effective adoptive immunotherapy against human cancer. However, two recent developments have changed the approach to treatment of cancer using immunological intervention. First was the large scale production of recombinant IL-2, previously known as T cell growth factor. Second was the observation that peripheral blood-derived lymphocytes cultured in IL-2 [lymphokine-activated killer (LAK cells)] become cytolytic toward a wide range of fresh neoplastic but not normal cells (46,85). LAK cells, together with high dose IL-2 as reported by Rosenberg and colleagues, have had some success in the treatment of metastatic human malignancies, notably melanoma and renal cell carcinoma (11,82). In some centers, the continuous infusion of cancer patients with lower doses of IL-2 alone without administration of *in vitro* generated LAK cells has yielded comparable therapeutic results.

The LAK phenomenon is mediated by a phenotypically diverse set of effector lymphocytes generated by incubation of peripheral blood leukocytes with IL-2 for 3 days. LAK effectors appear as mostly NK cells, but with time a more T-like phenotype emerges. The majority of LAK cells are derived from NK cells expressing the Leu 19 (NK H-1), but not CD3, surface marker. Peripheral blood CD3⁺ T lymphocytes appear to contribute little to the LAK phenomenon (47). However, under certain circumstances, a particular subset of CD3⁺ NK H1⁺ cells can also be activated by IL-2 and mediate LAK activity.

Recently, Rosenberg (82) reported that lymphocytes infiltrating into tumors could be expanded *in vitro* with IL-2 and used in adoptive immunotherapy. These tumor-infiltrating lymphocytes (TILs) had activity and tumor specificity superior to that of LAK cells. It remains unclear how much of the activity associated with TILs is due to CTL versus LAK and how useful these cells will be in the treatment of human cancers.

Ascites tumors provide an excellent model system to study TILs in experimental animals as well as in humans. TILs procured from the peritoneal cavities of animals that had undergone intraperitoneal allogeneic or syngeneic tumor graft rejection provide a rich source of small to medium sized effector CTLs capable of specific target cell lysis *in vitro* and tumor growth retardation *in vivo* (86). These potent CTLs are devoid of lytic granules, the lytic protein perforin, and BLT-esterase activity. They transform into granule-containing, cytolytic lymphoblasts within several days in culture in the presence of IL-2 (see Fig. 1) (87,88). This observation provides a clue to the cellular and possibly molecular basis for improved efficacy in the immunotherapeutic application of TILs—CTLs expanded in IL-2. Namely, the superb immunotherapeutic activity of IL-2 activated TIL against tumors may be related to their acquisition of cytoplasmic granules in response to IL-2. Proteases and cytotoxic proteins packaged in cytoplasmic granules of IL-2-transformed lymphocytes may be responsible for the antitumor as well as side effects resulting from the administration of large doses of IL-2 *in vivo*.

Tissue Damage in Autoimmunity

Cytocidal lymphocytes seem to play a significant role in inflicting tissue damage in certain autoimmune diseases. This discussion is limited to one autoimmune disease, namely, experimental autoimmune encephalomyelitis (EAE) in rodents. The disease, manifested by nerve conduction defects, can be induced by injecting the animal with myelin basic protein (MBP) or whole brain homogenate in complete Freund's adjuvant. EAE, characterized by massive infiltration of lymphocytes into the central nervous system, is a model system for inflammatory and demyelinating human disorders such as multiple sclerosis. T cells of the helper-inducer phenotype ($CD4^+$) have been implicated as the effectors of EAE, but the mechanism whereby they induce demyelinating damage in the brain, resulting in disease, is not known; furthermore, even the recent production and characterization of encephalitogenic $CD4^+$ T cell lines capable of inducing EAE (89) have not resolved this enigma. Furthermore, the proposed linkage between EAE and delayed-type hypersensitivity induced by $CD4^+$ effectors was recently contradicted by the demonstration that $CD4^+$ encephalitogenic T cells expressing IL-2 receptors can cause EAE in the absence of DTH (90). Recently, cytotoxic $CD4^+$ cells were implicated as causing the onset of symptoms of EAE, possibly by damaging blood vessels in the central nervous system. That Ia-restricted encephalitogenic T lymphocytes lyse autoantigen (MBP)-presenting astrocytes *in vitro* suggests that lymphocytes can directly induce brain damage (91). Studies with the Lewis rat encephalitogenic T cell line (Z1a) capable of inducing EAE in rats have shown substantial lectin (Con A)-dependent cytolytic activity against a wide range of target cells, confirming the proposition that $CD4^+$ Z1a encephalitogenic lines are indeed cytotoxic. Cytocidal lymphocytes have also been associated with a number of other autoimmune diseases involving lymphocyte infiltrates and tissue damage.

LYMPHOCYTE-TARGET CELL INTERACTION

Binding of Effector and Target Cells

Lymphocyte-induced target cell lysis involves a complex series of events, the first of which is quick specific or nonspecific binding of the CTL or NK to its target, resulting in conjugate formation (Fig. 2). Brondz and co-workers (92,93) originally established the immunological specificity of the physical interaction between CTLs and target cells by demonstrating depletion of cytolytic activity of alloimmune lymphoid cell populations incubated on target cell (macrophage) monolayers genetically similar to the immunizing cells. That this depletion was not due to specific inactivation of the effector cells was demonstrated independently by Golstein et al. (94) and by Berke and Levey (95), who showed that the effector lymphoid cells that adhered specifically to target cell monolayers

could be recovered. The CTLs thus collected were found to have an increased cytotoxic activity against the specific target cell employed for absorption. Later, Stulting and Berke (96) introduced poly-L-lysine (PLL)-fixed target cell monolayers, to study CTL-target cell binding and showed that Mg^{2+} but not Ca^{2+} was essential for specific CTL-target cell binding. A similar Mg^{2+} requirement was later demonstrated for NK-target cell binding. Since the monolayer absorption technique examines adhesion of lymphoid populations rather than of individual CTLs, answers to questions concerning the binding of individual CTLs could not be obtained. This has been resolved by the CTL-target conjugation method.

Conjugate Formation

Unequivocal specific binding of CTLs and target cells, resulting in conjugate formation (Fig. 2) was established and termed in 1975 (53,97). It is an early event in the multistep process of lymphocyte-induced lysis. Conjugate formation is induced simply by mixing CTL and target cells in a Mg^{2+} -containing medium (96), spinning to promote interaction, gentle resuspension to break up large cell clumps and weak (non-specific) interactions, and then microscopic examination. Guided by earlier CTL work, NK-target conjugation, and more recently conjugation of T_h cells with Ag-presenting cells, has also been observed. While specific conjugate formation by *in vivo* primed murine CTLs has been repeatedly shown (54), conjugate formation by human and murine CTL lines growing *in vitro* has been found to exhibit considerably less and sometimes even a complete lack of specificity. That conjugated lymphocytes are indeed functional cytotoxic effectors has been demonstrated using single-cell micromanipulation techniques (98). This finding has served as the foundation for a series of experiments aimed at answering questions regarding lymphocyte-target cell interactions at the individual cell level, rather than by extrapolating from population studies.

Specificity, Clonality, and Avidity of Lymphocyte-Target Conjugation

In vivo primed murine CTLs preferentially bind to and form conjugates with target cells displaying MHC determinants identical to, or cross-reacting with, the alloimmunizing cells (53,97). With *in vivo* primed CTLs from the peritoneal cavity of alloimmunized mice (PEL-CTL), the specificity of CTL-target cell conjugation is displayed at all CTL/target cell ratios examined. It appears that up to 40% of immune PEL are capable of specifically conjugating to the immunizing tumor cells. Five to ten percent of these alloimmune peritoneal cells bind nonspecifically to antigenically irrelevant cells (53). Specific binding probably involves the CTL T_i -CD3 receptor complex and

the target MHC class I Ag. The CTL CD8 (or CD4), LFA-1 and CD2 (T11, sheep red blood receptor, LFA-2) membrane determinants also contribute to intercellular binding (99). NK cells preferentially conjugate to NK-sensitive target cells, but neither the NK receptor nor the target determinants recognized by NK cells are known at the present time.

CTL conjugation behaves "clonally" since CTLs from animals immunized simultaneously by two antigenically dissimilar tumor allografts form only little heteroconjugates (i.e., one CTL bound to two different types of target tumor cells) (54). Heteroconjugates, however, can form in the presence of a mitogenic lectin, such as concanavalin A which enables binding of CTLs and third-party target cells. Brondz et al. (93) were the first to demonstrate the generation of distinct subpopulations of alloimmune CTLs against membrane Ag coded for by the MHC H-2D and H-2K subloci by differential immunoadsorption on cell monolayers. A "gene-dosage" effect in the induction of CTLs, as determined by conjugation, is suggested by the results presented in Table 1. Thus alloimmunization of mice with a single peritoneal tumor allograft differing from the host at one or two MHC haplotypes generated 19 and 29% specific conjugate-forming non-adherent peritoneal cells, respectively, while simultaneous intraperitoneal immunization with allogeneic tumors differing from the host at three or four MHC gene loci generated a total of about 48 and 60% conjugate-forming cells, respectively. Lower frequencies of conjugating effectors in a syngeneic intraperitoneal tumor system has been demonstrated (66).

In contrast to specific T cell receptor (Ti)-mediated tar-

get cell binding by *in vivo* primed murine CTLs, antigen specific CTL clones propagated *in vitro* often bind less specifically to target cells although they still kill specifically (99). Three distinct molecular species, namely, LFA1, CD2, and LFA3, appear to be involved in this nonspecific adhesion. The finding that the molecules involved in nonspecific adhesion are also intrinsic to specific CTL-target binding suggests that weak, Ag-nonspecific interactions precede specific interactions mediated by the CTL Ti receptor and the target cell surface Ag (99,100). Theoretical considerations suggest that relatively few receptor-receptor or receptor-ligand bonds may be necessary and sufficient to initiate firm adhesion between cells (101,102). It has been suggested that CD8 and CD4 molecules function to stabilize the interaction between CTL receptors and the corresponding target and/or stimulating cell class I and class II MHC Ags, respectively. Such stabilization may be required by CTLs possessing few and/or low-affinity receptors (103-105) since effector-target cell binding is an equilibrium process (106,107). The avidity of CTL-target cell junctions has been measured directly by determining the force required to separate a conjugated CTL from specific and nonspecific target cells, prior to the delivery of the lethal hit. Interestingly, the force required to break the bonds between specifically conjugated cells (1.5×10^4 dynes/cm²) is about 10 times greater than that required to separate a nonspecific lymphocyte-target cell pair (108). Hence most of the binding force and probably the energy holding a specific CTL and target cell together must come from Ti-CD3 interactions with MHC determinants of the target.

TABLE 1. Gene-dosage effect in CTL-target cell conjugation in alloimmunization

| PEL as a CTL source (MHC subloci) | Number of differing MHC subloci | % PEL in conjugation* | | | | | Nonspecific conjugation (highest %) | PEL in conjugation ^b per MHC sublocus (estimated) |
|--|---------------------------------------|-------------------------|--------------------------|-------------------------|-------------------------|------|---|--|
| | | Target cells | | | | | | |
| | | EL4 K ^D b | P815 K ^D d | ALB K ^D d | YAC K ^D d | | | |
| BALB/c anti-YAC (anti-K ^a) | 1 | 10.5 | 3.9 | 4.8 | 29.5 | 10.5 | 19/1 | |
| BALB/c anti-EL4 (anti-K ^b D ^b) | | 42.8 | 11.8 | 3.9 | 8.5 | 11.8 | | |
| C3H/eB anti-EL4 (anti-K ^b D ^b) | | 46.6 | 16.0 | 6.2 | | 16.0 | | |
| C57BL/6 anti-YAC (anti-K ^a D ^d) | 2 | 8.2 | | | 42.0 | 8.2 | 29/2 | |
| C57BL/6 anti-P815 (anti-K ^b D ^d) | | | 47.4 | 35.0 | | | | |
| C57BL/6 anti-ALB (anti-K ^b D ^d) | | | | 48.3 | | | | |
| C3H/eB anti-P815 (anti-K ^b D ^d) | | 10.8 | 39.5 | 29.6 | | 10.8 | | |
| BALB/c anti(EL4 + YAC) (anti-K ^b D ^b K ^a) | 3 | 40.5 | | | 17.5 | — | ~48/3 | |
| C3H/eB anti(P815 + EL4) (anti-K ^b D ^b K ^a D ^b) | 4 | 36.8 | 34.1 | | | — | ~60/4 | |

* CTL-target cell conjugates were formed by mixing 0.2×10^6 PEL with 10^6 fluorescein diacetate-labeled target tumor cells and scored under a fluorescence microscope.

^b Highest value of nonspecific conjugation was subtracted.

Targeting of Cytolytic Lymphocytes by Lectins and by Antibodies

The exquisite specificity of CTL-mediated lysis, as best demonstrated by MHC restriction of CTL recognition and lysis, can be changed drastically so that CTLs can *non-specifically* lyse virtually any target cell. This can be achieved (a) in the presence of mitogenic plant lectins, such as Con A, or PHA [lectin-dependent cytotoxicity (LDCC)] (32); (b) if the cells are subjected to mild oxidation (109) by periodate (IO_4^-) or by galactose-oxidase [oxidation-dependent cytotoxicity (ODCC)]; (c) by cross-linking the CTL and the target cell with antibodies against the CTL T_H receptor or receptor-associated structures such as CD3 (110) and even through surrogate target antibody inserted in the lymphocyte membrane in a mode that precludes interaction of the inserted antibody and membrane receptors of the effector lymphocyte (111); and (d) by cross-linking Fc-bearing cytotoxic cells to antibody-coated target cells (ADCC) (112).

Lectins are sugar-binding proteins and strong cell agglutinins. It is unclear why only certain plant lectins, notably Con A and PHA, stimulate polyclonal production of cytotoxic lymphocytes *in vitro* and mediate CTL–target cell interactions, resulting in nonspecific lysis. A putative, and unproven, lectin-induced *activation* step (not involving the TcR) following an initial *bridging* (conjugation) step have been proposed as the two signals required and delivered by mitogenic lectins in LDCC (113–115). While mixed cell aggregates of lymphocyte and target cells can be induced by most lectins, only mitogenic lectins appear to induce stable and effective CTL–target conjugation and lysis (Table 2). Furthermore, pretreatment of the target but not of the effector by Con A results in effective cytotoxicity (116). Con A functioning as a “bridge” would be

expected to have a bidirectional role, namely, to induce lysis by pretreated CTLs as well. Therefore it has been proposed that the fundamental mechanism of nonspecific recognition and lysis in LDCC may not be simply through “bridging” and “activation” of the bridged effector by the lectin. Rather it may be analogous to specific CTL–target cell interaction (116,117). That is, the CTL–Ag receptor(s) complex interacts with the target cell surface determinants (including MHC Ag) altered by mitogenic lectins (in LDCC) or oxidants (in ODCC) (116–118). These modifications of target cell surface determinants enable stable, nonspecific conjugate formation ultimately leading to lysis (Table 2). The analogy between specific and lectin or oxidant-dependent T cell lysis is strengthened by the fact that both LDCC and specific CTL-mediated killing are multiphasic, proceeding through a Mg^{2+} -dependent conjugation step, Ca^{2+} -promoted programming for lysis, and a final killer-cell-independent target cell disintegration stage. Furthermore, both specific CTL-mediated killing and antigen nonspecific LDCC or ODCC are similarly influenced by metabolic and cytoskeletal inhibitors, as well as by antibodies directed against CTLs and target cell surface components involved in CTL–target cell interaction (113,115–119).

Antibody-dependent cellular cytotoxicity (ADCC) has traditionally been attributed to “K” cells, macrophages, and NK cells, which belong to antibody-coated target cells (usually red blood cells) through FcR of the effectors. Targeting of cytotoxic lymphocytes (both CTLs and NK) by antibody against either effector or target cell surface determinants is a new, rapidly developing field of considerable theoretical and practical implication. In specific CTL-induced lysis, binding of Ag (or Ag–MHC complexes) on the target cell by the CTL $\alpha\beta$ chains of the heterodimer receptor (TcR) activates the CTL lytic machinery. However, T_H or CD3 specific antibodies attached to various target cells through their Fc portions and the FcR of the target can also trigger the lytic activity of CTLs, leading to lysis of the antibody-derivatized target (110). This reaction probably occurs because of the close physical association of T_H and CD3 molecules in the CTL membrane, as revealed by cocapping and coprecipitation experiments. (Fab')₂ fragments directed against T_H or CD3 do not mediate lysis due to physical proximity, since the Fc portion of IgG (FcR) is involved in the intercellular contact. T_H and CD3 antibody cross-linked to anti-target antibody have also been used to direct CTLs against certain targets. In another system CTL-induced specific lysis of an anti-CD3-producing hybridoma cell has been shown. These last two findings emphasize the role of T_H–CD3 triggering in inducing lymphocyte-mediated cytotoxicity and offer new, exciting means for targeting cytotoxic cells in tumor therapy and virus infection (120–124).

TABLE 2. Lysis and conjugate formation mediated by mitogenic and nonmitogenic lectins

| Lectin | Lysis ^a (%) | Number of conjugates ^b |
|---------------------------------------|---------------------------|--------------------------------------|
| Mitogenic | | |
| Con A (<i>Canavalia ensiformis</i>) | 46.0 | 64 |
| PHA (<i>Phaseolus vulgaris</i>) | 77.0 | 68 |
| LCA (<i>Lens culinaris</i>) | 87.0 | 57 |
| Nonmitogenic | | |
| PNA (<i>Arachis hypogaeae</i>) | 4.3 | 1.5 |
| SBA (<i>Glycin max</i>) | 14.0 | — |
| PWM (pokeweed mitogen) | 6.2 | 7.5 |

^a To measure cytotoxic activity, polyclonally activated splenocytes of DBA/2 origin were reacted with ⁵¹Cr-labeled EL4 as targets. Mitogenic lectins were tested at 2 to 10 $\mu\text{g}/\text{ml}$; nonmitogenic lectins at 20 to 50 $\mu\text{g}/\text{ml}$. CTL/target cell ratio was 10:1. Incubation time was 90 min at 37°C.

^b To measure conjugate formation, peritoneal exudate CTLs were mixed with irrelevant EL4 or P815 cells (1×10^6 cells each), centrifuged at 170g for 10 min at room temperature, resuspended, and the number of conjugates/0.5 μl was determined.

Quantification of Lymphocyte–Target Cell Interaction Resulting in Conjugate Formation

The term conjugates refers to clusters of effector lymphocytes firmly bound to target cells (Fig. 2) (53). In the

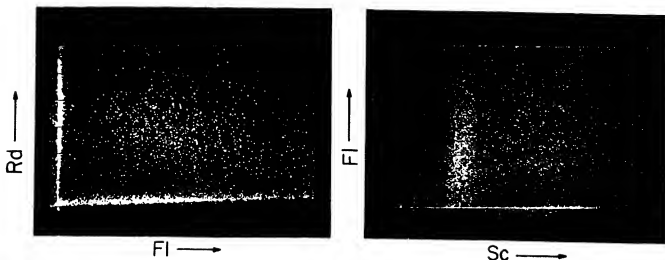


FIG. 6. Analysis of lymphocyte-target cell conjugation by flow cytometry. Fluorescein (Fl) labeled BALB/c anti-EL4 PELs were conjugated with rhodamine (Rd)-labeled target cells (EL4). Left: Rd versus Fl showing conjugates in the diagonal area and nonconjugated effector and target cells on the ordinates. Right: Fl versus scatter (Sc) showing low Sc particles (Fl-PEL); high Sc, low fluorescence particles (EL4); and large, labeled particle (conjugates). (Adapted from Berke, ref. 125.)

conjugation method introduced in 1975 (53,97), suspensions containing CTLs and target cells are cocentrifuged at room temperature to induce conjugation. After thorough yet gentle resuspension, the fraction of lymphocytes bound to target cells is determined in hemacytometers. CTL and target cells can be distinguished by size or by the use of fluorescent dyes. A similar conjugation technique has been applied to NK and T_h cells. To overcome the limitations of microscopic scoring of lymphocyte-target conjugates such as subjectivity, sizes of populations scored, and speed, a cytofluorometric procedure employing a fluorescence-activated cell sorter (FACS) has been developed (125,126). The method is based on the simultaneous monitoring of single-color fluorescence of either prelabeled CTLs or target cells and scatter, or of double-color fluorescence of the conjugated effector and target cells (Fig. 6). The cytofluorometric method has been developed further to sort out specific CTL-target cell conjugates (49), to score conjugate-forming NK cells, and to study single-cell kinetics of lysis.

Competitive inhibition of CTL-target conjugation, assessed by monitoring the impact of unlabeled target cells on conjugate formation between CTLs and fluorescently labeled target cells can be used to evaluate and compare cell surface Ags involved in CTL-target cell interaction (127). Unlabeled homologous target cells lower the frequency of fluorescent target cells in conjugation, proportional to their fraction of the overall target cell population. This method has certain advantages over "cold" target cell inhibition of NK or CTL-mediated lysis, where recycling of effectors influences the linearity of inhibition.

LYMPHOCYTE-MEDIATED CYTOLYSIS

Assessment of Lysis

Target cell lysis following interaction with effector cytotoxic cells can be determined by release of incorporated

radioactive molecules, uptake of dyes which are excluded by intact viable cells, or by end-radiolabeling of the residual cells. Testing the plating efficiency of target cells after interaction with effectors evaluates both cytotoxic and cytostatic effects but is seldom used since it is cumbersome. In 1968, Brunner et al. (128) modified the ^{51}Cr -release assay, long employed to measure survival time of red blood cells as well as complement-induced lysis of nucleated cells, to measure lymphocyte-mediated cytotoxicity. This simple procedure has been used since with virtually no modification since it correlates well with cell lysis and can be used to monitor large numbers of samples. Once incorporated into cells (as $\text{Na}_2^{51}\text{CrO}_4$), spontaneously or actively released ^{51}Cr is not reincorporated, most probably due to changes in the ionic and oxidation state of chromium, binding to aminosugars, peptides, and other cell constituents. For many target cells, the release of the gamma emitter ^{51}Cr from prelabeled target cells provides a good estimate of their viability, correlating quite well with the uptake of trypan blue or eosine dyes. With certain target cells, particularly during extended lytic assays, high spontaneous release of ^{51}Cr complicates interpretation of the results, in which instance alternative radioisotopic procedures (such as the release of the gamma-emitting amino acid ^{75}Se -selenomethionine or of ^{111}In) or other procedures to determine target lysis must be employed.

With ^{51}Cr (isotopic) release assays, percent lysis is often calculated as follows:

$$(\text{experimental cpm} - \text{spontaneous cpm}) / (\text{total releasable cpm} - \text{spontaneous cpm}) \times 100$$

Although most of the incorporated ^{51}Cr binds to releasable low molecular weight cellular components (3,000 daltons and less), about 25% of all that incorporated binds tightly to high molecular weight cellular components and is nonreleasable even at 100% cell lysis induced by re-

peated freeze-thawing or by exposure to detergents or acids. Depending on the assay conditions, cell type, and assay duration, spontaneous ^{51}Cr release values of 5 to 25% can be obtained. In addition some of the labeled target cells present during lytic assays are not conjugated and occasionally spontaneous ^{51}Cr release due to spontaneous cell death or passive leakage may be substantial (up to 25 to 40%). Hence to compare the lytic activity of populations exhibiting different levels of conjugation, it is important to know the kinetics of lysis solely attributable to effector cells (50). The expression lytic units (LUs) is often used to compare the lytic activity of effector populations (13). One LU is defined as the number of effectors that produce 37% lysis in a unit of time, as deduced from lytic data obtained with serially diluted effectors. Because the extent of lysis is a function of both the concentrations of effectors and of the target, as well as of time, the determination and comparisons of LUs must be done cautiously.

Lysis induced by individual effectors conjugated to target cells can be assessed directly by a variety of methods (review in ref. 129). These include single-cell manipulation by micropipettes and phase contrast microscopy (98), examination of the viability (by dye exclusion) of lymphocyte-target cell conjugates incubated in hemacytometers or in agar in the presence of eosine or trypan blue, or recently, flow cytometry of effector-target cell conjugates (125,126,130). Under conditions minimizing effector cell recycling and formation of new CTL-target cell interactions, by using, for instance, highly viscous media (dextran) (131) or by dilution in excess medium, the number of specifically lysed target cells can provide a rough estimate of CTL frequencies.

Dynamics of Lymphocyte-Target Binding, Lysis, and Recycling

Lymphocyte-mediated cytotoxicity is a multistep process initiated by the binding (conjugate formation) of an effector lymphocyte (CTL or NK) to a target cell, delivery of the lethal hit, target cell dissolution, and recycling of the effector (Fig. 7) (57,132). Conjugate formation is a rapid, receptor-mediated process, promoted by cocentrifugation of effector and target cell mixtures (53). The fact that binding is optimal at room temperature, where virtually no lysis occurs, enables the study of this binding stage on its own (132). Dual parameter fluorocytometry has been used to demonstrate that conjugate formation follows first-order kinetics with a half-time of 1.4 min (126). Balk et al. (106,107) have demonstrated that CTL-target cell binding is an equilibrium process. They have also shown the reversal of specific cell-cell adhesion between allogeneic CTLs and ^{51}Cr -labeled target cells. The rate of this reversal appears to depend on the relative affinity of the CTLs to the bound versus free target cells.

Rate of lysis is defined as the number (or density) of target cells lysed by a given number of effectors in a unit of time. Depending on the effector-target system employed, incubation periods of varying lengths are required to detect significant cytotoxicity. For example, CTL can

usually lyse normal or neoplastic lymphoreticular target cells within 1 to 4 hr and even less, whereas fibroblast monolayers may require 12 to 24 hr to lyse. Some CTL populations (e.g., alloimmune peritoneal exudate) kill faster than others and generally the rate of NK-induced lysis is slower than that of CTLs. Additional kinetic considerations are as follows: (a) The rate of lysis induced by either CTLs or NK cells is proportional to the concentration (density) of both effectors and target cells (Fig. 8) (57,133). (b) CTLs can recycle and kill at the same rate (98,134) at least two to three times (Table 3), whereas functional recycling of NK cells requires reactivation of the effectors (135) although evidence for two cycles of killing by some NK cells has been presented and there may not be a clear distinction between NK cells and CTLs in this regard. (c) Some target cells are lysed as early as 5 to 10 min and others as late as 2 to 3 hr after onset of interaction with the effector (conjugate formation). (d) There is a short lag phase (5 to 20 min) before initiation of ^{51}Cr release (13,133). The lag period is not affected by increasing the effector/target cell ratio. However, a delay in ^{51}Cr release from target cells has been observed in both syngeneic systems (136) and when CTL-induced lysis of some target cells (e.g., leukemia EL4 of C57BL/6 mice) occurs in the absence of Ca^{2+} in the medium (137). (e) Temperature has a marked effect on both the rates of conjugation and lysis, although maximum conjugation occurs at room temperatures at which virtually no lysis is detected (53,57). Interestingly, lowering the temperature at an advanced stage of CTL-target interaction (after the delivery of the lethal hit) will completely halt lysis; however, this activity will return to its original rate when the temperature is returned to 37°C (18,138). These results indicate the existence of an intermediate state of a "hit" target which is committed to but not fully lysed.

Escape of CTLs from Self-Annihilation and the Polarity of Lysis

Considerable evidence shows that effector CTLs are not inactivated as a result of deploying their lytic machinery, but that they can recycle to participate in a new lytic interaction (see Table 3) (98,134). Thus any theory on the mechanism of at least CTL-mediated lysis must be compatible with the unidirectionality of the killing process as well as with the recycling of the effector. This does not seem to be the case with human NK cells. Temporary loss of human NK activity as a result of interactions with the NK-sensitive target K562 has been reported although killing activity of the NK population is restored by an IL-2-dependent mechanism (135). If the lytic signal is induced by contact with the effector only and does not involve secretion of a pore-forming toxin(s) into the intercellular gap, then obviously CTL self-destruction would not occur. On the other hand, how effector cells would avoid self-killing, while deploying a "secreted" lytic protein(s), constitutes a dilemma. Hypothetical protective mechanisms have been proposed, but supportive evidence for their existence is not currently available with the exception of homologous restriction factor (HRF) de-

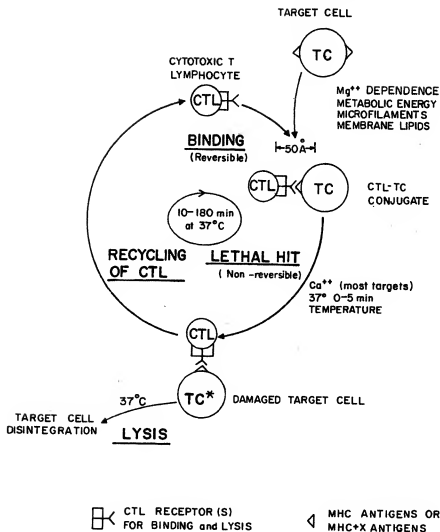


FIG. 7. Lymphocyte-mediated cytotoxicity. (From Berke, ref. 54, with permission.)

scribed by Muller-Eberhard and colleagues. Sparing of the effector CTL is a particularly important issue since, in 1974, Golstein (139) demonstrated that CTLs are not "immune" to attack by showing that B anti-C CTLs can be inactivated (as determined by ability to lyse C target cells) when exposed to A anti-B CTLs (Fig. 9). Interestingly, cloned CTL lines and *in vivo* primed CTLs appear to be more resistant to lysis or inactivation induced by CTLs or by lytic granules as opposed to lysis mediated by antibody plus C (140-143).

That CTLs kill only in the direction of (target) recognition has been deduced from the selective inactivation of only B anti-C CTLs during coincubation with A anti-B CTLs (Fig. 9) (144), as well as from the refractiveness of A anti-B effectors to "reverse" lysis induced by (A × B) F1 anti-X effectors upon lysis of X target cells (Fig. 9) (145). When studied at the individual conjugate level, unidirectional lysis has been observed even during interaction of mutually immunized CTL populations (A anti-B with B anti-A) (146) although at the population level, as determined by the ⁵¹Cr-release assay, bidirectional lysis is observed. Unidirectional lysis at the population level has also been demonstrated in lectin (Con A)-dependent,

nonspecific killer anti-killer lymphocytotoxicity (116). It appears that effector lymphocytes will not express their lytic potential unless their Ag specific receptor complex is occupied by Ag or triggered by Ti-CD3 antibody and will only lyse target cells that are receptor bound (but see ref. 111). Although some conflicting findings exist, bystander cells are usually not lysed, nor are cells bound to killer cell surface Ag other than the Ti receptors or Ti-associated structures such as CD3 (110). However, lysis mediated through CD2 recognition has been reported (the sheep red blood receptor of T cells) (147). The unidirectional lysis of A anti-B or B anti-A CTLs in the course of A anti-B-B anti-A interaction (Fig. 9) may be the result of a head-to-tail type interaction, possibly due to the asymmetric distribution of either CTL surface receptors or cytoplasmic constituents or both. Unidirectional lysis of such mutually immunized CTLs would also occur if the CTL that bound first or more effectively survived the interaction. When a single CTL is bound simultaneously to a number of target cells, the targets are lysed sequentially, not simultaneously (148). This finding also supports the idea of a focal delivery of the lethal hit.

In summary, the lytic event in CTL-mediated lysis ap-

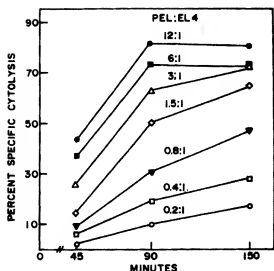


FIG. 8. Time course of lysis of EL4 cells incubated with BALB/c anti-EL4 immune peritoneal exudate cells. PEL:EL4 indicates effector-to-target cell ratios. (From Berke et al., ref. 17, with permission.)

pears to be contingent upon engagement of the CTL T_H-CD3 receptor(s) with a relevant target cell MHC complex Ag and may actually be mediated either by the receptor itself or by associated cell surface components located close by on the membrane or by a secreted component. The receptor-Ag interaction could thus result in target membrane perturbation leading to lysis, or the killer cell receptor could thereby regulate entry into the target of a toxic factor or formation of a membrane channel resulting in cell death. Third-party and bystander experiments have discounted the role of a nonspecific soluble mediator released into the macroenvironment around the killer cell. However, a soluble mediator released upon receptor activation, active only in the intercellular microenvironment and rapidly degraded, thus appearing unidirectional, must be considered.

TABLE 3. Recycling of CTLs: Single-cell analysis by micromanipulation

| Experiment | Killers ^a tested | Killers ^b that bound a second time | Killers ^b that lysed a second time |
|------------|-----------------------------|---|---|
| 1 | 10 | 2 | 2 |
| 2 | 12 | 4 | 3 |
| 3 | 16 | 3 | 3 |
| 4 | 10 | 9 | 9 |

^a BALB/c anti-EL4 peritoneal exudate killers were isolated with a micropipette after lysing their conjugated EL4 cells, as determined by trypan blue uptake.

^b Isolated killer cells were placed in contact with intact EL4 cells with the aid of a micropipette and tested for repeated binding and killing activity.

Data adapted from Zagury et al. (98).

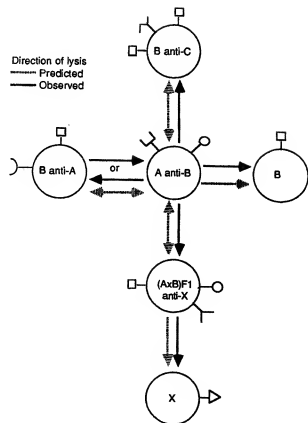


FIG. 9. Unidirectionality of lysis and the role of the CTL receptor in lysis. A anti-B, B anti-A, B anti-C, and (A × B) F₁ anti-X represent specifically immunized CTLs. γ, γ₁, γ₂, γ₃ represent receptors for C, B, A, and X antigenic determinants, respectively. □, γ₁, γ₂, γ₃ represent B, A, and X antigenic determinants, respectively.

Contact Region of Cytotoxic and Target Cells

Electron microscopy of CTL-target cell conjugates has revealed extensive interdigitization of the plasma membranes at the contact region (Fig. 2) (149-151). Formation of fingerlike structures at the intercellular contact zone has also been observed by microcinematography under UV light, of live fluorescein diacetate-labeled CTLs conjugated to live unlabeled target cells. Effector cell projections involved in conjugation contain a network of fine fibrillar material and are devoid of ribosomes, granules, and other obvious cellular organelles. The interdigitization suggests that membrane-folding forces are generated at zones of CTL-target cell contact. This is consistent with the energy dependence of conjugate formation and the requirement of an intact cytoskeletal system (54), unlike the binding of antigen to receptor-bearing B lymphocytes which can occur at 4°C and in the presence of azide. Immunofluorescence microscopy showed that the CTL contact region is enriched with actin but not myosin (152). Anti-tubulin immunofluorescence and EM showed that CTLs usually bind to target cells through a membrane region proximal to the CTLs' microtubule organizing center (MTOC) and centrioles (Fig. 10) (153). A similar



FIG. 10. Localization of the microtubule-organizing center (MTOC) in lymphocyte-target conjugates by tubulin antibody. The MTOC of the CTL (arrow) is proximal to the area of contact, while the MTOC of the target cell is randomly oriented. Bars represent 10 μ m; magnification is 1,200 \times . (Adapted from Geiger et al., ref. 153.)

observation was made later with NK-target cell conjugates (154). It is unclear whether cytoplasmic polarity of the MTOC is related to delivery of the lytic signal proper or to adhesive properties of the cell membrane adjacent to the MTOC, which favor formation of stable intercellular contacts. Proximity of CTL and NK contact regions to the MTOC and centrioles may be related to the general role of the centriole in the effector cell movement toward the target, once contact occurred. For example, it has been shown in motile cells that the centrioles are located in front of the nucleus toward the leading edge of the cell membrane. The membrane in this area of motile cells exhibits an increased protrusive and deformational potential, which may render it more compatible to the formation of stable intercellular contacts. Another CTL organelle found localized in the vicinity of contact regions is the Golgi complex (98,155), suggesting but not confirming the role of a secretory process in conjugate formation and/or target cell lysis or lymphokine secretion. Rearrangement of cytoplasmic granules and other cellular organelles toward the NK-target cell binding site has also been reported (156,157). Based on time-lapse cinematography it has been suggested, but remains to be shown, that after realignment these granules fuse with the membrane and their contents are released into the junctional area of the effector-target conjugate (158) and then are involved in lysis.

Intercellular Communication: Effector Molecules and Secretory Processes in Lymphocytotoxicity

Cell-to-cell communication mediated by intercellular channels is common in organized tissues. Formation of cytoplasmic junctions between effector lymphocytes and target cells, although repeatedly suggested in the past,

was not observed in ultrastructural and tracer studies (149,159). Likewise, freeze-fracture studies did not reveal clear-cut alterations in the distribution pattern of "intramembrane particles" in effector-target contact regions, suggesting but not proving that intercellular membrane fusion does not occur. Thus although unique and temporary communicating membrane substructures may form, failure to detect communicating junctions between the CTL and its target or to demonstrate, by EM, effector cell granules in the process of fusion strengthens the notion that at least certain lymphocyte-induced cytotoxic interactions are strictly contactual and do not involve transfer of material from effector to target through cytoplasmic continuity or granule fusion with the membrane.

The existence of a lytic, or nonlytic, intracellular or secreted material of effector cell origin is not sufficient in itself to establish its role in lymphocytes inducing lysis. However, in recent years, evidence has accumulated suggesting a Ca^{2+} -dependent exocytosis mechanism in at least NK-induced and possibly also CTL-induced lysis. This evidence includes the following:

1. Release of proteoglycans from cytoplasmic granules of human NK cells during target cell lysis (160) and of serine protease(s) during CTL-induced lysis (161).
2. Rearrangement of the cytoplasmic granules, organelles, MTOC, and Golgi of CTLs during lysis (157).
3. Very low NK activity in Chediak-Higashi patients and in beige (bg/bg) mice that both possess mutations causing abnormal lysosomal structure (162). Interestingly, these same beige mice exhibit CTL activity, thus showing the existence of two separate types of lytic mechanisms (NK cells and CTLs).
4. Temporary inactivation of NK cells (but not of CTLs) after lytic contact with target cells (135), suggesting functional secretion by NK but not CTL.
5. Reduction of NK (but not CTL) activity by strontium, known to induce leukocyte degranulation.

6. Inhibition of NK lytic activity by lysosomotropic agents that interfere with lysosomal structure and function, such as chloroquine, NH_4Cl , as well as by drugs (such as Monensin) which affect Golgi-mediated secretion. However, the effects of Monensin on CTL/NK-mediated lysis are complicated by the significant inhibition of CTL-target conjugation (G. Berke and D. Rosen, unpublished results) as well as by the fact that this drug inhibits vesicle fusion to the Golgi apparatus and probably does not influence exocytosis of prepackaged lytic granules as proposed for NK- and CTL-induced lysis. Furthermore, lysis in the absence of Ca^{2+} in the medium, a condition which prohibits granule exocytosis, as well as perforin-induced cytotoxicity has been demonstrated (137,163).

Subcellular and Soluble Cytolytic Factors in Cell-Mediated Cytotoxicity

There are indications for a secretory process in lymphocytotoxicity and several lytic factors have been obtained from the culture medium of resting or activated cytotoxic lymphocytes or extracted directly from the cells. Cytotoxic lymphocytes can produce two different types of cytotoxic effector molecules, namely, granule-associated proteins that can induce prompt, nonspecific cell lysis and other non-granule-associated secreted proteins that can cause protracted (12 to 24 hr) but selective lysis.

NK Cytotoxic Factor(s) (NKCF)

This slow-acting factor(s) is released from murine, rat, or human NK cells during their interaction with NK susceptible targets. Release of NKCF can also be induced by mitogens. Because the factor(s) retains both the species and target specificity of NK cells, it is believed to be involved in target cell lysis mediated by NK cells (Fig. 11) (164). NKCF is absorbed by susceptible target cells through putative NKCF binding receptors, possibly cell surface glycoproteins/glycolipids. Its internalization appears to induce irreversible damage to the target by an unknown mechanism, resulting in target lysis without further exposure to NK cells. Clearly more work is required to establish the precise molecular nature and function of NKCF in NK-induced lysis.

Lymphotoxin (LT) (TNF- β)

Activation of B or T lymphocytes with Ags or mitogenic lectins can lead to production of LT (also called TNF- β), a tumor necrosis factor (TNF- α)-like molecule of 171 amino acids (~18 kd) which can nonspecifically lyse certain sensitive target cells (165–167). However, *in vivo* (74) and *in vitro* (168) experiments with bystander cells to detect diffusible effector molecules have indicated that LT may not be involved in CTL-mediated lysis. Furthermore,

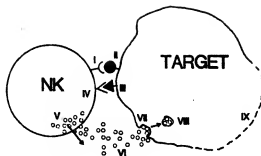


FIG. 11. Model proposed for the mechanism of lysis induced by NK cells. (I) NK recognition structure. (II) NK target structure(s). (III) Stimulating target cell structure. (IV) NK receptor. (V) Release of NKCF. (VI) Released NKCF. (VII) NKCF binding site. (VIII) NKCF processing. (IX) Target cell death. (Adapted from Wright and Bonavida, ref. 164)

LT antibodies which retard LT activity in a delayed-type hypersensitivity reaction do not affect CTL-induced lysis (168). However, the possibility has not been excluded that LT or another lytic product is secreted into the intercellular space or transferred from CTL to target cells through specialized junctions, following specific CTL-target cell conjugation.

Tumor Necrosis Factor (TNF- α)

This toxic factor is found in the serum of animals infected with *Bacillus Calmette-Guérin* (BCG) and endotoxin (167,169,170). TNF- α is capable of inducing hemorrhagic necrosis of murine tumors, particularly when injected into the tumor lesion. It is a nonglycosylated protein of 157 amino acid residues (~17 kd). TNF- α is produced by macrophages, and macrophage-induced lysis is blocked by TNF- α antibodies. Neither TNF- α nor TNF- β antibodies blocks lysis induced by NK cells. TNF- α is structurally homologous to LT (TNF- β) and exhibits 30% amino acid homology. TNF- α and LT interact with cells through a specific class of high-affinity receptors ($K_d < 10^{-10}$ M), the number of which is increased by interferon. Both TNF- α and TNF- β (LT) are cytotoxic to only a narrow range of cells, with L929 cells being the most susceptible target.

Lytic Granules, Cytolysin/Perforin, and Serine Proteases

NK cells and some but not all CTLs, particularly those cultured with IL-2 or responding to stimulation by Ags/mitogens or IL-2, contain defined cytoplasmic granules, which have been observed by EM (Fig. 12), isolated, purified on Percoll gradient, and analyzed (43,171–173). These cytoplasmic granules contain the Ca^{2+} -dependent lytic protein(s) perforin/cytolysin, hydrolytic lysosomal enzymes, and several distinct serine proteases and proteoglycans (~200 kd). Ca^{2+} -dependent exocytosis of

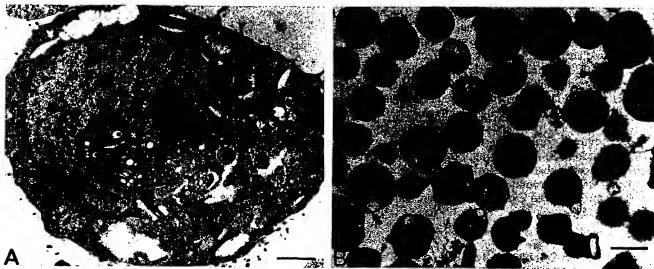


FIG. 12. Electron micrographs of a granular CTL and its isolated granules. A: A CTL-2 cell. Note abundance of osmiophilic granules (gr). Bar represents 1.2 μ m. mit, mitochondria. B: Purified granules extracted from CTL-2 cells. Bar represents 526 nm. Electron microscopy by D. Rosen.

granule contents has been proposed to be involved in target cell lysis induced by cytotoxic lymphocytes. At least one and possibly two proteins of 70 to 75 kd (reduced) are present in purified granules extracted from both NK cells and some CTLs that contain such granules. They are antigenically related to the ninth component of complement (C9) (171,174), but unlike C9, cytolysin/perforin in itself causes rapid and Ca^{2+} -dependent lysis of a variety of target cells, including red blood cells as well as nucleated cells which are NK resistant (175). Both human and mouse perforin have been recently cloned, the genes exhibiting 30% homology to C9. Cytolysin/perforin induce the disintegration of liposomes as determined by rapid release of internalized carboxyfluorescein (43). When polymerized, perforin forms characteristic hollow tubular structures (I.D. 150 to 200 Å) spanning the membranes of the target cells (171). Negatively stained and examined by EM, these structures appear as "rings" embedded in the membrane (Figs. 13A and 14). Functional transfer of perforin from granulated effector CTL to target cell has been inferred in one report from the immunofluorescence of perforin antibody applied to CTL-target cell conjugates (171). Curiously, the antibodies directed against perforin, which effectively block lysis induced by purified perforin or perforin-containing lytic granules, do not block lysis induced by *in vivo* primed CTLs, hence direct proof for the involvement of perforin/cytolysin in CTL-induced lysis is lacking.

A few distinct CTL specific serine esterases have been discovered both at the gene and at the protein levels (176-179). These include CTLA1 (also termed CCPI or granzyme B) and CTLA3 (H factor, granzyme A). Lytic cytoplasmic granules can be extracted from CTL-NK cell lines and appear to be the intracellularly localized storage organelle of these enzymes. The enzymes are released upon specific CTL-target cell interaction, resulting in

lysis (179,180), but their role, if any, in inducing lysis is as yet unknown (181). Recently we observed transient expression of serine-proteases during primary but not secondary CTL response *in vivo* (Gardyn and Berke, *unpublished*), suggesting that CTL transiently acquire granules containing serine-proteases in the course of their primary stimulation, probably under the influence of IL-2. Hence protease activity of CTL correlates with their stage of differentiation rather than cytotoxic activity. Two newer CTL genes, CTLA-A2 α and CTLA-A2 β , appear to have the pro-region of a cysteine protease whose function in inducing killing is also unknown at the present time.

THE "LETHAL HIT" AND THE MECHANISM OF LYSIS

Early Studies

Several theories have been proposed for the nature of the lethal hit and the mechanism of lymphocyte-induced lysis (reviews in refs. 2-4,182-184). The apparent sequential release of cellular constituents from the affected target according to molecular size, combined with the protective effects of high molecular weight dextrans (185,186), supported the theory of small lymphocyte-induced target membrane damage, ultimately leading to colloidal-osmotic swelling followed by target cell membrane rupture (187). Alternatively, the firm adhesion of CTLs to target cells was proposed to cause tangential shear force on the target cell membrane, leading to target cell damage and lysis (188,189). Yet another theory proposed the activation of a membrane-bound phospholipase resulting in removal of one fatty acid from phosphatidylcholine, thereby causing formation of lysophosphatidyl-

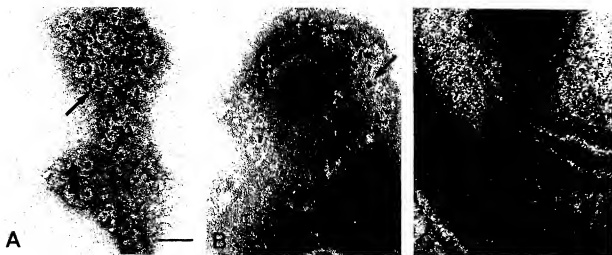


FIG. 13. Membrane-bound "ring" structures in immune cytotoxicity. **A:** Membranes following lysis induced by hyperimmune serum + complement (BALB/c anti-EL4 serum plus EL4 target cells). Typical rings are seen (arrow). Bar represents 43.8 nm (A), 55.5 nm (B), and 47.6 nm (C). **B:** Membranes following NK-induced lysis (human peripheral blood LGL plus K562). Typical rings are seen (arrow). **C:** Negatively stained membrane isolated after CTL-induced lysis (BALB/c anti-EL4 PEL plus EL4 target cells). No rings are seen. Electron microscopy by D. Rosen.

choline (lyssolecithin), a strong detergent and a potent cytolytic agent, capable of destroying the integrity of the target cell membrane. Phospholipases generally do not attack the phospholipids of intact plasma membranes; however, they may cleave membrane phospholipids in the presence of membrane-perturbing factors, such as detergents or a basic snake venom peptide and possibly during CTL-target cell interaction. As plasma membrane fractions from stimulated lymphocytes were found to lyse target cells, it was suggested that physical contact with the effector cell membrane in itself may be sufficient to induce target lysis. However, linkage between CTL recognition and lytic activities, as manifested by lack of lysis of A anti-B upon interaction with B anti-C CTLs (144), suggests that the mere close apposition of CTL and target cell membranes, although required, is insufficient to cause lysis (Fig. 9).

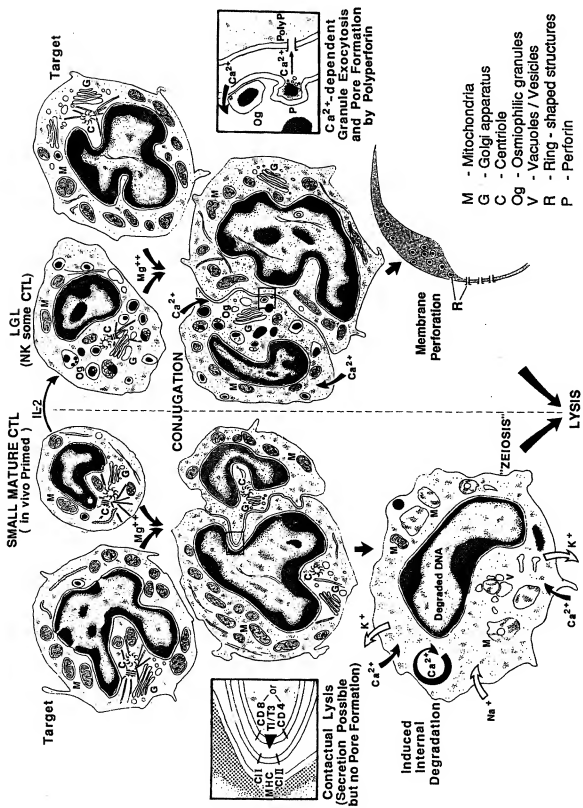
Current Studies and Theories

Due to some common characteristics and the same end result (cytolysis), there is a recent tendency to propose a common lymphocytotoxic mechanism, regardless of the effector cell-type involved (43,171,190). Such an example is the proposed mechanism of lymphocytotoxicity mediated by exocytosis of lytic granules and formation of 10- to 20-nm pores in the target cell membrane. However, the elucidation of certain defined, prelytic events and the careful analyses of *in vivo* primed effector cell populations devoid of lytic granules and perforin suggests the existence of more than one lytic pathway in lymphocytotoxicity (191-194). Furthermore, target lysis induced even by granule-containing lymphocytes (CTLs or NK cells)

can occur in the absence of granule secretion (163,195). Interestingly, lytic granules can be induced in highly potent *in vivo* primed peritoneal exudate CTLs devoid of such granules, upon cultivation *in vitro* in IL-2 (87,88). Taken together, the recent results suggest that either granule exocytosis is not involved in inducing lysis, or that the effector cells can lyse targets by two different lytic mechanisms. Currently, two nonmutually exclusive theories exist to explain the mechanism whereby various CTLs and NK cells induce lysis: (a) that a pore-forming protein(s), packaged in cytoplasmic granules and secreted upon CTL or NK interaction, induces lysis of the target (43,171,174) and/or (b) that a transmembrane stimulatory signal(s) delivered to the target upon receptor-mediated effector-target interaction induces internal disintegration of the target (191-194). The two theories, which probably apply to different effector cell types, are schematically illustrated in Fig. 14 and are discussed next.

Granule Exocytosis and Formation of 100- to 200-Å Pores in the Target Membrane

This theory (43,171) stems from the observation that NK cells and certain CTLs possess lytic cytoplasmic granules and the discovery of complement-like lesions on membranes of lysed cells. It has been suggested that target cell binding to a specific NK cell and to certain CTL-membrane receptors induces a secretory process in the effector cell which results in the contents of the lytic cytoplasmic granules being released. It is these lytic components in the localized environment which have been proposed to cause target cell lysis. NK- and CTL-mediated lysis would thus be the end result of perforation of target cell membranes. Indeed negative staining electron microscopy of lysed targets in some experiments has



shown protein-lined transmembrane holes (internal diameter, I.D., 100 to 200 Å) which appear as membrane-bound "rings" (Figs. 13A,B and 14). They are believed to be structurally and functionally analogous to target cell lesions produced by the membrane attack complex of complement (Fig. 13A) (171,196). Cytoplasmic granules of CTLs and NK cells, which contain the lytic protein(s) perforin/cytolysin (~70 kD), lysosomal enzymes, and serine protease(s) activity, have been proposed to be the origin of the ring-forming material(s). Ca^{2+} has been claimed to be necessary for (a) the induction of exocytosis of the lytic granules and (b) inducing polymerization of the lytic protein perforin (perforin $\xrightarrow{\text{Ca}^{2+}}$ polyperforin), believed to be involved in the perforation of the target membrane (see Fig. 14, right-hand box). However, the demonstration of CTL-induced cytotoxicity in the absence of Ca^{2+} (137) would seem to invalidate this mechanism and suggest an alternative pathway.

Effector Cell Triggering of Target Disintegration Not Initiated by Pore Formation

Several observations are not consistent with a single mechanism of CTL- and NK-induced lysis, mediated solely by perforation of the target cell membrane with 100- to 200-Å "holes" by effector granule constituents:

1. Perforin/cytolysin has been detected in nonlytic cells, while certain *in vivo* primed, highly potent CTLs derived from the peritoneal cavity of alloimmunized mice lack perforin or lytic granules or both, and they do not induce complement-like "rings" in the lysed target membrane (Fig. 13C)

(43,88,191,197-199). Also, most cloned CTLs do not seem to have detectable perforin (199).

2. CTL-induced lysis of some target cells can occur in the absence of Ca^{2+} in the medium (137), a condition in which no exocytosis of granule constituents is demonstrable (163,195), and perforin/cytolysin is nonlytic to cells since perforin-induced toxicity is strictly Ca^{2+} -dependent (43).
3. The *prelytic* increase in cytosolic Ca^{2+} in target cells (137,191) and the disintegration of the target DNA into 190-bp units prior to ^{51}Cr release (194,200,201) are difficult to reconcile with a lytic mechanism initiated by the formation of "holes" of I.D. 100 to 200 Å in the target membrane!
4. The release of ^{51}Cr , nicotinamide, amino isobutyric acid, and even ^{86}Rb from CTL-damaged target cells is temperature dependent and is fully arrested at 7°C and even 20°C (18,138), while leakage from damaged targets through putative protein-lined "holes" (I.D. 10 to 20 nm) should be only minimally affected by temperature, if at all.
5. Antibodies against lytic granules and/or components thereof (such as perforin/cytolysin) block lysis induced by cytolytic granules but not by *in vivo* primed peritoneal exudate CTLs and other CTLs (202).

The alternative

In view of these intriguing findings, an alternative lytic mechanism must exist. Lymphocyte-induced internal disintegration of the target cell (autolysis) has been suggested by some investigators (192,194). We shall now discuss the possible nature of such an inductive signal delivered by

FIG. 14. Schematic illustration of two currently proposed pathways in lymphocyte-mediated lysis. Contactual lysis inducing self-disintegration (*left*) and granule exocytosis resulting in pore formation (*right*). Killing by contactual lysis (*left side of scheme*) is effected by *in vivo* primed, small to medium sized (7 to 10 µm) CTLs devoid of lytic granules and perforin and which exhibit only background levels of BLT-esterase activity. These effectors are MHC-restricted, T1/T3-positive CTLs. In the presence of Mg^{2+} , these CTLs bind target cells, thus forming conjugates. The CTL MTOC and Golgi complex are oriented toward the contact area, which is characterized by membrane interdigitation. Under permissive ion and temperature conditions, a cascade of events is initiated in the target, culminating in zellolysis and cytotoxicity. These include target membrane derangement caused by CTL binding, which results in K^{+} efflux (depolarization), and an increase of cytosolic Ca^{2+} (from external/internal stores). Ca^{2+} may be involved in inducing prellytic DNA degradation, damage to the nucleus and mitochondria, activation of ATPases, and ATP depletion, blebbing, and membrane damage culminating in zellolysis. The effector cell can recycle without reactivation. In the lytic granule exocytosis pathway (*right side of scheme*), the killer is a large (10 to 20 µm) granular lymphocyte (certain CTLs and NK cells). Some of these effectors are T1/T3⁺, MHC-restricted, while others (including NK cells) lyse nonspecifically. These effector cells are characterized by lytic cytoplasmic granules containing perforin and BLT-esterase(s). When presented with an appropriate target, the granular effector cell undergoes a series of events, similar to those with agranular killers, to form interdigitated contact with the target. This is followed by external Ca^{2+} -dependent granule exocytosis and release of a pore-forming protein(s) (perforin/cytolysin) into the intercellular gap. The released perforin undergoes Ca^{2+} -dependent polymerization to form a protein-lined hole (I.D. 100 to 200 Å) perforating the target cell membrane. These lesions appear as ring-shaped structures in negative-staining EM. Other granule and nongranule components [NK cytolytic factor (NKCF), $\text{TNF-}\alpha$, $\text{TNF-}\beta$] may contribute to target lysis. This effector cell type requires reactivation before it can interact with and lyse another target cell.

the killer and how target lysis is achieved (scheme, Fig. 14, left-hand side). It has been proposed that CTL-mediated lysis is initiated by multiple submicroscopic molecular derangements in the target membrane structure (191) upon lymphocyte-target interaction. Membrane derangements may result from direct contact of the target MHC and/or other cell surface determinants with the CTL T α -CD3 complex (Fig. 14, left-hand box) and involve other membrane-bound (e.g., CD8 or CD4) or secreted lymphocyte effector molecule(s) (192). Under permissive temperature and ionic conditions, a multitude of such intercellular contacts would induce membrane depolarization as measured by $^{86}\text{Rb}^+$ (a K^+ analog) efflux from the target, simultaneously with the delivery of the lethal hit (131,203). Subsequent permeability changes observed in the target following contact with CTLs probably reflect progressively failing ion pumps, which are only initially capable of controlling K^+ efflux (depolarization) and Na^+ and Ca^{2+} influx through the deranged (depolarized) membrane.

The outcome of any low-level chemical, physical, or immunological (complement- or CTL-induced) damage to targets depends on that cell's ability to compensate for and/or repair the damage inflicted. Small perturbations in ion fluxes are dealt with by enhanced outward ion, mainly Na^+ and Ca^{2+} , pumping activity, energy metabolism, and oxygen consumption by the affected cell. Substantial, or small but persistent, derangements of the cell membrane can exhaust energy resources involved in ion pumping. Cessation of outward Na^+ pumping ultimately leads to net water influx, colloidal-osmotic cell rupture, and target disintegration. On the other hand, the prelytic, postlethal hit loss of cellular K^+ occurs with the concurrent entry and accumulation of Na^+ . Although the transmembrane ion concentration gradients are thus dissipated, the intracellular concentration of colloids (proteins) is still higher than that present externally, and the cells thus begin to swell slowly. Hence disruption of the Donan equilibrium may facilitate net uptake of water, swelling, lysis, and release of high molecular weight compounds (131,203,204).

The role of cytosolic Ca^{2+} in inducing cellular injury

The above sequence of events does not account for certain prelytic effects (preceding ^{51}Cr release) characteristic of lymphocyte-induced cytotoxicity. These include DNA disintegration, cytoplasmic streaming, blebbing, and finally "zeiosis" (cell boiling). The prelytic elevation of cytosolic (and probably nuclear) Ca^{2+} levels (which can be detected within minutes of lymphocyte-target cell interaction) has been suggested to be an essential prelytic event, inducing internal disintegration processes in the target cell which culminate in zeiosis (137,191,192). Increase in Ca^{2+} may contribute to DNA fragmentation (194,200), through triggering of Ca^{2+} -dependent topoisomerases and nucleases, which induce uncoiling and fragmentation of the DNA, respectively. Target cell protease activity induced or enhanced by Ca^{2+} could damage cy-

toskeletal elements resulting in bleb formation (205) and finally zeiosis. Mitochondria were shown to be one of the first cellular organelles affected in the course of lymphocyte-mediated cytotoxicity. Energy production by mitochondria could be suppressed by Ca^{2+} -induced damage to mitochondrial structure/function, affecting generation of ATP and thus the function of ATP-fueled ion pumps. Ca^{2+} enhancement of phospholipase activity may induce damage to the plasma and internal membranes. Ca^{2+} activation of major cellular ATPases, such as actomyosin, could lead to massive ATP and phosphocreatine depletion, further suppressing Na^+/K^+ -ATPase (sodium pump) activity, already compromised by the depolarized membrane and decreased mitochondrial ATP production (191,192).

IL-2-Induced Acquisition of Cytocidal Granules, BLT-Esterase and Perforin mRNA by Lymphocytes and the Two Pathways of Lymphocytotoxicity

The demonstration of complement-like "holes" (I.D. 100 to 200 Å) on membranes of targets lysed by effector lymphocytes and of lytic granules and perforins, in certain cytotoxic lymphocytes (immature CTL *in vivo*, LGL or CTL cultured in IL-2), led to the suggested mechanism of lytic granule exocytosis and a common terminal step in lymphocyte and complement-induced lysis. However, neither the formation of complement-like "rings" in targets were caused by, nor have lytic granules, perforin/cytolysin, or serine esterases been found in either highly potent, mature peritoneal exudate CTLs (day 11 after primary or day 5 after secondary immunization), in cytotoxic hybridomas generated from them (PEL hybridomas), or in a number of other CTL lines (88,197,198). We have recently found that upon incubation *in vitro* in the presence of IL-2, the small *in vivo* primed cytotoxic PELs transform into large, dividing cytotoxic T cells (PEL-blasts), which express authentic PEL specificity in short-term lytic assays (87,88). PEL-blasts, in contrast to the small *in vivo* primed PELs, possess massive quantities of lytic granules (Fig. 1), serine esterase activity (Table 4)

TABLE 4. Expression of protease (BLT-esterase) activity in various CTL

| Cells | BLT-esterase activity ^a |
|--|------------------------------------|
| CTL-2 (IL-2 dependent, <i>in vitro</i>) | 6.00 |
| PEL-CTL (4 days after <i>in vivo</i> priming) ^c | 0.540 |
| PEL-CTL (11 days after <i>in vivo</i> priming) | 0.228 |
| PEL-CTL (5 days after <i>in vivo</i> priming) | 0.019 |
| PEL-CTL hybridoma | 0.056 |
| PEL-Blasts (IL-2 dependent, <i>in vitro</i>) | 6.8–47.0 ^b |
| Normal spleen | 0.222 |
| EL4 leukemia | 0.220 |
| P815 leukemia | 0.386 |
| L1210 leukemia | 0.018 |

^a OD 412 nm/ 1×10^6 cells/15 min.

^b Various cultures.

^c Transient expression of BLT-esterase during primary but not secondary PEL-response (Gardyn and Berke, *in preparation*).

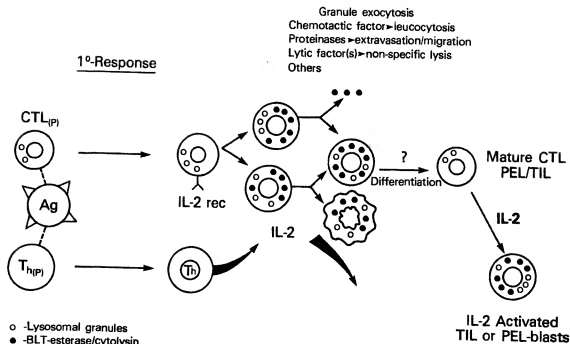


FIG. 15. The function of granules in lymphocyte activation modulation by IL-2.

and perforin mRNA (Berke, Podack and Lowry, *in preparation*). IL-2 also appears to modulate the expression of BLT-esterase and cytoplasmic granules in the course of CTL differentiation (Fig. 15), as we have found transient expression of BLT-esterase activity during primary CTL differentiation in the peritoneal cavity (Gardyn and Berke *unpublished*). Hence, granule and protease expression in CTL correlates with their stage of differentiation rather than cytotoxic activity. Granules may play a role in CTL trafficking and in response to antigen, as well as in the elimination of responding cells. The proposed mechanisms involving exocytosis of lytic granules may apply to killing induced by granule-containing effectors, such as LGLs and CTL cultured in IL-2, but not mature granule-free CTL such as PEL (Fig. 5). Thus there appear to be two distinct pathways of lymphocytotoxicity, only one of which involves secretory lytic granules.

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EXHIBIT G

immunodominant region of the autoantigen MBP. This may provide insight into the molecular mechanisms of MS and help in the design of new specific therapeutic approaches.

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 - Sequences of MBP peptides: MBP(1-46-102): DPVVFHFHFGFQVDFGKLVDPVMBP(146-168): FKGVDAQGTLSSKFLEGGDR. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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 - GGAAGGATTC; Vβ20, 5' ACCGACAGGCT-GAAGGACGGGGGCTCCAGC; Ca, 5' GOGA-CAGGACGAGGCGCTTGCTGTAGGACAC; Cα probe, 5' TTCTGATGCTCAAACACAGGAG-CTCGGG; Vβ17 leader, 5' AGCAACAGGTG-CTCTGAGTGTGGTGGTCTT; and Igλ, 1, 5' CC-CTCGGCGGCAAGATCTCTATTGTAGGA; J.
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- 28 February 1990; accepted 30 April 1990
- ## A Receptor for Tumor Necrosis Factor Defines an Unusual Family of Cellular and Viral Proteins
- CRAIG A. SMITH,* TERRI DAVIS, DIRK ANDERSON, LISABETH SOLAM, M. PATRICIA BECKMANN, RITA JERZY, STEVEN K. DOWER, DAVID COSMAN, RAYMOND G. GOODWIN
- Tumor necrosis factor (TNF) and TNF-(B) bind surface receptors on a variety of cell types to mediate a wide range of immunological responses, inflammatory reactions, and anti-tumor effects. A cDNA clone encoding an integral membrane protein of 461 amino acids was isolated from a human lung fibroblast library by direct expression screening with radiolabeled TNF-α. The encoded receptor was also able to bind TNF-β. The predicted cysteine-rich extracellular domain has extensive sequence similarity with five proteins, including nerve growth factor receptor and a transcriptionally active open reading frame from Shope fibroma virus, and thus defines a family of receptors.
- TUMOR NECROSIS FACTOR α (TNF-α, cachectin) and β (TNF-β, lymphotxin)** are structurally and functionally homologous proteins secreted by activated macrophages and lymphocytes, respectively (1). These cytokines have pleiotropic activities in vitro and in vivo, including cytotoxic effects against tumors and virus-infected cells, stimulation of interleukin-1 secretion, stimulation of prostaglandin E2 and collagen production, inhibition of lipogenic gene expression in adipocytes, and stimulation of various immune effector cells (2). Clinical interest has focused on TNF because it appears to be a common mediator of inflammation, endotoxin-induced shock (1), and the wasting syndrome commonly observed in chronic infections and neoplastic disease (3). TNF receptors appear on virtually all somatic cells (1), and generally the ligands cross-compete for binding (4), suggesting they share a common receptor. As an aid to studying the TNF system in molecular detail, we isolated a cDNA clone of the receptor.
- The SV40-transformed human lung fibroblast cell line WI26-V44 was used as a source of mRNA for construction of a cDNA library. This cell line binds both TNF-α and -β and displays multiple affinity classes; approximately 23,000 binding sites per cell (N) were detected with ¹²⁵I-TNF-α that could be fit to two affinity classes, low ($K_D = 0.16 \pm 0.10$ nM⁻¹ N₁ = 19,200 +
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A Receptor for Tumor Necrosis Factor Defines an Unusual Family of Cellular and Viral Proteins

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Tumor necrosis factor- α and β (TNF- α and TNF- β) bind surface receptors on a variety of cell types to mediate a wide range of immunological responses, inflammatory reactions, and anti-tumor effects. A cDNA clone encoding an integral membrane protein of 461 amino acids was isolated from a human lung fibroblast library by direct expression screening with radiolabeled TNF- α . The encoded receptor was also able to bind TNF- β . The predicted cysteine-rich extracellular domain has extensive sequence similarity with five proteins, including nerve growth factor receptor and a transcriptionally active open reading frame from Shope fibroma virus, and thus defines a family of receptors.

TUMOR NECROSIS FACTOR α (TNF- α , cachectin) and β (TNF- β , lymphotxin) are structurally and functionally homologous proteins secreted by activated macrophages and lymphocytes, respectively (1). These cytokines have pleiotropic activities in vitro and in vivo, including cytotoxic effects against tumors and virus-infected cells, stimulation of interleukin-1 secretion, stimulation of prostaglandin E2 and collagen production, inhibition of lipogenic gene expression in adipocytes, and stimulation of various immune effector cells (2). Clinical interest has focused on TNF because it appears to be a common

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The SV40-transformed human lung fibroblast cell line WI26-VA4 was used as a source of mRNA for construction of a cDNA library. This cell line binds both TNF- α and - β and displays multiple affinity classes; approximately 23,000 binding sites per cell (N) were detected with ^{125}I -TNF- α that could be fit to two affinity classes, low ($K_d = 0.16 \pm 0.10 \text{ nM}^{-1}$; $N_s = 19,700 \pm$

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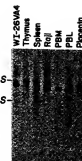
To whom correspondence should be addressed.

4,800) and high ($K_{d2} = 6.2 \pm 3.9 \text{ nM}^{-1}$, $N_2 = 3,000 \pm 1,400$) (Fig. 1A). TNF- β binds with lower affinity than TNF- α and the ligands cross-compete for binding (Fig. 1B). Double-stranded cDNA was synthesized by standard procedures, inserted into the mammalian expression vector pDC302 (5) and a TNF receptor clone isolated by a direct expression approach. Plasmid DNA from about 1000 *Escherichia coli* (DH5 α) transformants were pooled, transfected into COS cells, and screened by contact autoradiography (6), which detects positive pools by the ability of those COS cells expressing TNF receptor inserts to bind ^{125}I -labeled TNF- α . After screening 175,000 clones, one positive pool (#737) was obtained, subdivided, and converged to a single clone in two cycles of this procedure. By autoradiographic plate binding (6), the pure clone when transfected into COS cells expressed a receptor that bound both ^{125}I -TNF- α and β ; binding of either ligand was completely inhibited by a 200-fold excess of the same or homologous unlabeled cytokine (7). Quantitative *in situ* binding studies of the COS-expressed receptor with ^{125}I -TNF- α agreed with these results and showed the binding to be complex (Fig. 1C). As with the native

W126-VA4 receptor, the recombinant COS receptor displayed both low ($K_{d1} = 0.18 \pm 0.06 \text{ nM}^{-1}$) and high ($K_{d2} = 10.1 \pm 1.0 \text{ nM}^{-1}$) affinity classes for ^{125}I -TNF- α . TNF- β bound with lower affinity and competitively inhibited ^{125}I -TNF- α binding (Fig. 1D). Thus, ligand binding properties of both the native and recombinant receptor appear similar. The origin of the multiple affinity classes for TNF- α is unclear. Indeed, most workers (1, 4, 8, 9), but not all (10), have reported monophasic Scatchard plots for TNF- α . However, TNF- α is predominantly a homotrimer (11) and therefore intrinsically capable of multivalent binding. In one report (12), differential biological effects could be related to biphasic binding of TNF- α . We not necessarily sharing a common origin, multiple affinity classes are a common feature of many receptor systems (13).

The isolated TNF receptor cDNA was used as a probe to analyze the mRNA expressed in a variety of cell lines and tissues (Fig. 2). A single size class of transcripts of ~4.5 kb was detected in W126-VA4, Raji cells (a B lymphoblastoid line), LPS-stimulated peripheral blood monocytes (PBM), induced peripheral blood T cells (PBL), and

Fig. 2. RNA blot analysis of TNF receptor mRNA. Polyadenylated RNA (3.5 μg) was used from each source, except placental tissue (5 μg total RNA). PBL were cultured for 6 days in IL-2 and OKT3 monoclonal antibody, then restimulated for 8 hours with concanavalin A (Con A) and PMA (6). RNA was fractionated on a 1.1% agarose-formaldehyde gel, blotted onto Hybond-N (Amersham), and hybridized with a labeled antisense RNA probe prepared from the 630-bp Nor 1-Bgl II fragment of the TNF receptor cDNA that had been subcloned into a Bluescript plasmid (Stratagene). Filter hybridization and washing conditions were as described (5). Variable exposure times were used in preparing the figure.



placental tissue. A transcript of slightly larger size (~5.0 kb) was detected in thymic tissue, and splenic tissue contained transcripts of both size classes. The origin of these differences is not clear, but the presence of TNF receptor transcripts in these different cells is consistent with the near ubiquitous distribution of the receptor.

The 3.7-kb insert of clone 737 was subcloned and sequenced (5) (Fig. 3). The cDNA contains a string of adenines at the 3' end and an upstream consensus polyadenylation signal. The discrepancy between the size of the isolated cDNA and that of the transcripts estimated from Northern analysis may be due to a deficiency of 5' sequences in this clone. It is also possible that alternative polyadenylation signals are utilized. Upstream of the polyadenylation site is a 299-bp segment that has homology to the Alu family of repetitive sequences (14). The sequence contains a single large open reading frame encoding 461 amino acids with features typical of an integral membrane protein (15). The initiating methionine precedes 22 hydrophobic residues characteristic of a leader sequence; the most probable cleavage site (16) predicts Leu²⁵ as the mature NH₂-terminus. Another hydrophobic region of 30 amino acids is located between residues 258 and 287, bordered by charged residues at either end (Asp²⁵⁷ and Lys²⁸⁶⁻²⁹⁰), consistent with a transmembrane segment that makes a single helical span. Immediately upstream of this element is a region of 57 amino acids rich in threonine, serine, and proline residues. Such a composition is indicative of O-linked glycosylation sites containing sialic acid and is found in similar extracellular regions of several receptors, including those for nerve growth factor (NGF) (17) and low density lipoprotein (LDL) (18). The NH₂-terminal 162 amino acids (positions 39 to 200) are rich in

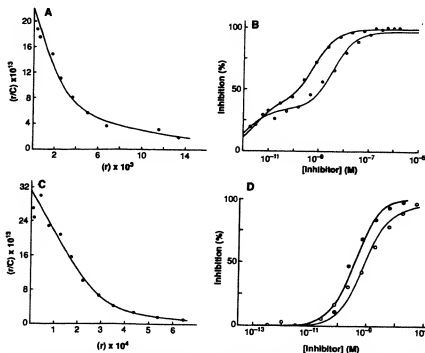


Fig. 1. TNF binding characteristics of native and recombinant TNF receptors (31). (A) Direct binding of ^{125}I -TNF- α to W126-VA4 cells (Scatchard coordinate system). (B) Inhibition of ^{125}I -TNF- α binding to W126-VA4 cells by unlabeled TNF- α (●) and TNF- β (○). TNF- α inhibition: K_{i1} (low affinity) = $1.6 \pm 0.2 \text{ nM}$; K_{i2} (high affinity) = $0.8 \pm 0.1 \text{ pM}$. TNF- β inhibition: K_{i1} (low affinity) = $0.29 \pm 0.06 \text{ nM}$; K_{i2} (high affinity) = $1.3 \pm 0.6 \text{ pM}$. (C) Direct binding of ^{125}I -TNF- α to recombinant (COS) TNF receptor. (D) High affinity site inhibition of ^{125}I -TNF- α binding to recombinant (COS) TNF receptor by unlabeled TNF- α (●) or β (○). K_{i1} (a) = $6.7 \pm 2.9 \text{ nM}$; K_{i1} (b) = $3.3 \pm 0.8 \text{ nM}$. C, free concentration of TNF (molar); r, molecules of TNF bound per cell. All parameter values are \pm standard error. Data fit to one or two site models as described (32).

cysteines (22 residues) and also contain two potential N-linked glycosylation sites. The receptor terminates in a cytoplasmic domain of 174 amino acids, rich in serines (18%), six of which are contiguous. Five cysteines and one potential N-linked glycosylation site are also present in this domain.

A computer search of several sequence databases (19) queried with the entire 439-residue sequence of the mature TNF receptor revealed five proteins with striking similarity: human and rat NGF receptor, CD40,

cDNA clone 4-1BB, and T2 (Fig. 4). Four of these are transmembrane proteins, two of which are known receptors (for human and rat NGF). CD40 is a B cell-localized surface antigen, found also on neoplastic cells of epithelial origin, that becomes phosphorylated in the cytoplasmic domain after binding the CD40-specific monoclonal antibody G28-5 (20). Clone 4-1BB was identified as a murine cDNA from induced helper and cytolytic T cell clones (21). Both molecules have been suggested to be cytokine recep-

tors for unidentified ligands. All identity between these four proteins is localized to the cysteine-rich regions of the extracellular domains; no homology was detected between the TNF receptor cytoplasmic domain and any proteins in the database. T2 is a transcriptionally active open reading frame from the Shope fibroma virus (SFV), a poxvirus that produces invasive malignancies in newborn rabbits (22). Although dominated by 22 conserved cysteines, the alignment is also reinforced by other conserved amino acids, particularly tyrosine, glycine, and proline. Thus, the extracellular domains of these molecules, presumably heavily disulfide bonded, probably share a common structural motif. Central to this motif would appear to be repeating homologous domains. Several groups have shown that the cysteine-rich regions of NGF receptor and CD40 can be resolved into either pseudo twofold repeats of about 80 amino acids or pseudo fourfold repeats of about 40 residues (17, 20). Similar repeats can be shown with the TNF receptor and T2, consistent with all these genes having arisen by duplication and divergence from a common gene. Since both NGF and TNF are oligomeric, repeating substructures in their receptors may aid in binding and predicts that the putative ligands for CD40 and 4-1BB may also be oligomers. The net charge associated with the cysteine-rich domains of these family members varies (-19 for NGF receptor; +1 for TNF receptor), which may be related to ligand specificity. Presumably, it is this NH₂-terminal region that contains the TNF binding site. Multiple lines of evidence have localized the (apoptonein B) ligand binding site of the LDL receptor to the NH₂-terminal (60-kD), cysteine-rich

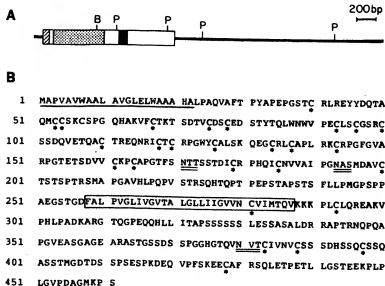
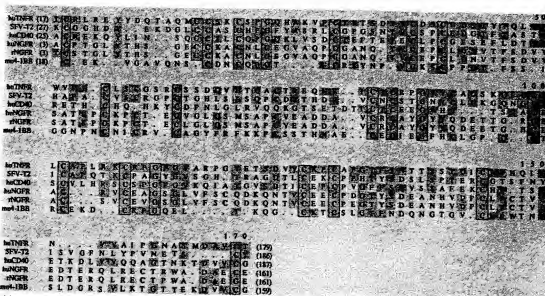


Fig. 3. Sequence of the human TNF receptor cDNA clone. (A) Schematic representation and restriction map of the cDNA. The entire coding region is boxed. The leader is hatched, the cysteine-rich region is shown stippled, and the transmembrane segment is solid. B = Bgl II, P = Pvu II. (B) The deduced amino acid sequence of cDNA coding region. The leader region is singly underlined, the transmembrane domain is shown boxed, potential N-linked glycosylation sites are doubly underlined, and cysteines are identified by an asterisk. The entire nucleotide sequence is available upon request and has been deposited at GenBank, accession number M32315.

Fig. 4. Sequence similarities among the TNF receptor superfamily. Consensus

alignment of residues from the cysteine-rich regions of human TNF receptor (huTNFR), T2 open reading frame of Shiga fibroin virus (SFV-T2), human CD40 (huCD40), human and rat nerve growth factor receptor (huNGFR and rNGFR), and murine cDNA clone 4-1BB (mur4-1BB). Numbers at NH₂- and COOH-termini refer to residues as cited in publications describing cDNA cloning (17, 20, 21, 22); numbers at top right of each block mark residues from NH₂-terminus at top left. Shaded residues reflect those common to huTNF receptor and at least one other protein. Cysteines are in



domain (18).

Sequences containing cysteine-rich repeats are present in a number of proteins, including the CD18 adhesion molecules (23), epidermal growth factor (EGF) precursor, *Drosophila notch* protein, the *new* oncogene, and the external domains of receptors for LDL, EGF, and insulin (18, 24). Although many of these proteins show homology to each other, we detect little similarity to the TNF receptor. Optimal alignments of family members using the National Biomedical Research Foundation (NBRF) ALIGN program (19) show the strongest similarity is between the TNF receptor and T2, with a score of 19 standard deviations (SD) above the mean score for an ensemble of randomly permuted molecules of the same lengths and amino acid composition. ALIGN scores greater than 3.0 are considered significant and indicate common ancestry. Almost 40% of the residues are identical, approaching the conservation level between many murine and human cytokines and their receptors (25). Slight variants of T2 may also exist in other poxvirus family members, and some of these viruses are strongly immunosuppressive (22). Although T2 possesses a signal peptide sequence, the molecule appears to lack a hydrophobic segment typical of transmembrane regions, suggesting that T2 may be a soluble entity secreted from virally infected cells. Thus, perhaps T2 may bind TNF, or another cytokine, serving to locally dampen the host immune response. The protective effects of such a "soluble receptor" would no doubt confer a selective advantage to the pathogen. CD40, however, is also similar to this TNF receptor (38.5% amino acid identity, 15.2 SD), yet does not bind TNF- α when expressed in COS cells at high levels in an immunoreactive form (26). TNF receptor is more distantly related to 4-1BB and NGF receptor (9.0 and 12.3 SD, respectively).

The signal transduction mechanism of TNF is unclear. The receptor cytoplasmic domain, as with other family members, shows no similarity with known proteins, including the cytoplasmic domain of the human T cell interleukin-1 (IL-1) receptor (6), despite the fact that TNF and IL-1 mediate many common biological activities (7). The TNF receptor expressed in COS cells does not bind radiolabeled human IL-1 α or - β , nor does the recombinant human IL-1 receptor bind TNF (7). No sequences present are typical of tyrosine kinases, protein kinase C, or phosphorylation sites corresponding to substrates for these kinases (27). The cytolitic activity of TNF, however, appears to depend on the presence of a 200-kD protein distinct from the receptor, and with which it comodulates (28).

Several groups have characterized TNF binding proteins from urine. Uromodulin is a renal glycoprotein that binds IL-1, IL-2, and TNF- α with high affinity, but does not inhibit ligand binding to their respective receptors and shows no sequence similarity to the TNF receptor reported here (29). Two groups have recently reported purification and sequencing of soluble TNF- α binding proteins from urine with molecular weights of 27 to 30 kD (30). However, the NH₂-terminal sequence of these proteins is not found in the predicted sequence of clone 737. TNF- α receptors on myeloid cells are probably different from those on cells of epithelial origin (8). An 80-kD form of the receptor contains O- and N-linked carbohydrate; a 60-kD form lacks O-linked carbohydrate, possesses a different form of N-linked carbohydrate, and displays different tryptic peptide maps. Monoclonal antibodies to these two receptors also do not cross-react. The receptor we have described may correspond to the 80-kD form. Affinity cross-linking of the recombinant receptor using either ¹²⁵I-TNF- α or - β shows a single species of 80 kD (7). Because the calculated protein is 46 kD, carbohydrate appears to be attached, and both O- and N-linked glycosylation sites are present in the sequence.

The availability of a full-length cDNA clone for a human TNF receptor will now permit detailed studies into the molecular mechanisms by which ligand-receptor interactions produce the pleiotropic effects of this important cytokine. Soluble, recombinant forms of this receptor may also be produced to explore the clinical value of TNF inhibition in pathological settings.

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25. The CD40 and human TNF receptor clones were separately transfected into COS cells (CD40-COS, TNF-COS) and expression was monitored by radiolabeled ligand binding using contact autoradiography (6). CD40-COS clearly and specifically bound ¹²⁵I- α 28 antibody, but did not bind ¹²⁵I-TNF- α (0.2 nM). ¹²⁵I- α 28 binding was not inhibited by a 500-fold molar excess of unlabeled TNF- α . Similarly, TNF-COS specifically bound ¹²⁵I-TNF- α and - β but not ¹²⁵I- α 28 (0.2 nM).
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31. COS cells were transfected with the vector pDC302 containing the TNF receptor cDNA insert (clone 737) or control vector lacking insert as described (5, 6). For quantitative *in situ* binding studies, transfected COS cells were replated (24 hours after transfection) into six well trays (CoStar) and analyzed 48 hours later at near confluence (6×10^5 cells per well). COS monolayers were washed once with phosphate-buffered saline (PBS), then incubated with 125 I-TNF- α at various concentrations in binding media [RPMI 1640, bovine serum albumen (10%), NaN₃ (0.1%), 20 mM Hepes, pH 7.4] at 4°C for 2 hours. Free 125 I-TNF- α was determined by counting gamma emissions in the supernatant. Monolayers were then washed once with ice-cold RPMI, detached with 0.1% trypsin in PBS, and counted to determine bound ligand. Nonspecific ligand binding was determined by inclusion of a 200-fold molar excess of unlabeled ligand. Inhibition assays used 125 I-TNF- α at 0.2 nM. Data were analyzed and theoretical curves plotted as described (6, 32). TNF- α and TNF- β (R&D Sciences) were radiolabeled using Iodogen (Pierce) to a specific activity of 2×10^{15} cpm/nmol (4). Radiolabeled TNF- α gel filtered as a single peak with an apparent molecular weight of 55 kD (7), consistent with a trimeric status (11).
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"The good news is we have the human genome. The bad news is the computer alphabetized it."